INVESTIGATION ON DIRECT *IN VITRO* SHOOT REGENERATION FROM AERIAL STEM EXPLANT OF GINGER (*Zingiber officinale* Rosc.) AND ITS FIELD EVALUATION

Thesis submitted to the

University of Calicut

For the award of degree

of

Doctor of Philosophy

(BOTANY)

by

Lincy A K

University of Calicut

Calicut, Kerala, India

2007

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Date: 21.8.07

CERTIFICATE

This is to certify that the thesis entitled "**Investigation on direct *in vitro* shoot regeneration from aerial stem explant of ginger (*Zingiber officinale* Rosc.) and its field evaluation**" submitted to the **University of Calicut** by Ms. **Lincy A.K.** for the award of the degree of **Doctor of Philosophy** in **Botany** is a bonafide record of research work carried out by her under my supervision and guidance. No part of the work has formed the basis for the award of any other degree or diploma previously.

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DECLARATION

I hereby declare that the thesis entitled "**Investigation on direct *in vitro* shoot regeneration from aerial stem explant of ginger (*Zingiber officinale* Rosc.) and its field evaluation**" submitted by me for the award of the degree of **Doctor of Philosophy** in **Botany** of the **University of Calicut** contains the results of bonafide research work done by me at Indian Institute of Spices Research, Calicut, Kerala, under the guidance of Dr. B Sasikumar, Sr. Scientist, Crop Improvement and Biotechnology Division, Indian Institute of Spices Research, Calicut. This Thesis or part of it has not been submitted to any other university for the award of any other degree or diploma previously. All sources of help received by me during the course of this study have been duly acknowledged.

Lincy A. K.

Calicut

21.8.2007

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Lincy A.K.

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Abbreviations

2,4-D	2,4- Dichlorophenoxy Acetic Acid
µl	Microliter
µM	Micromole
@	At the rate
°C	Degree Celsius
AA	Ascorbic Acid
AC	Activated Charcoal
AM	Apical Meristem
AOAC	Association of Official Analytical Chemists
ASTA	American Spices Trade Association
BA	Benzyl Adenine
BAP	6-Benzyl Amino Purine
bp	Base pair
C	Callus regenerated plants
CD	Critical Difference
CFU	Colony Forming Unit
CL	Coleoptile
cm	Centimeter
CP	Conventionally propagated plants
CR	Coleorhiza
CRD	Completely Randomized Block design
CTAB	Cetyl Trimethyl Ammonium Bromide
CV	Coefficient of Variation
Dicamba	3,6 – Dichloro-*o*- anisic acid
DNA	Deoxyribose Nucleic Acid
DNTPs	Dinucleotide Triphosphate
EDTA	Ehtylene Diamine Tetra Acetic Acid
Fig	Figure
g	Gram
GA_3	Gibberellic Acid
GC	Gas Chromatography
GE	Globular Embryo
hr	Hour
IAA	Indole 3- Acetic Acid
IBA	Indole 3-Butyric Acid
Kg	Kilogram
Kinetin	6-furfuryl Amino Purine
LS	Longitudinal Section
M	Molar
mg	Milligram
mgl^{-1}	Milligram per liter
mm	Millimeter
MP	Micropropagated plants

MS Medium	Murashige and Skoog Medium
mt	Metric tones
NAA	α – Naphthalene Acetic Acid
nm	Nanometer
NS	Non Significant
PCR	Polymerase Chain Reaction
PE	Primary embryo
pH	Negative logarithm of Hydrogen iron concentration
ppm	Parts per million
PTM	Primary Thickening Meristem
R	Root
RA	Root Apex
RAPD	Random Amplification of Polymorphic DNA
RAS	Rhizome of aerial stem derived plants
RBD	Randomized Block Design
RC	Rhizome of callus regenerated plants
RCP	Rhizome of conventionally propagated plants
RNA	Ribo Nucleic Acid
rpm	Rotation per minute
RVB	Rhizome of vegetative bud regenerated plants
SA	Shoot Apex
SB	Shoot Bud
SC	Scutellum
SE	Secondary Embryo
SH medium	Schenk and Hildebrandt medium
SN	Scutellar Notch
SU	Suspensor
TAE	Tris Acetic Acid EDTA
TBA	Tertiary Butyl Alcohol
TDZ	N-Phenyl-N-1, 2, 3-thidiazol-5' – yl-urea
TLC	Thin Layer Chromatography
TS	Transverse Section
UV	Ultra Violet
V	Volume
VAM	Vesicular Arbascular Micorrhiza
Var.	Variety
VS	Vascular System.
W	Weight
w/v	Weight/Volume

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Introduction

1. Introduction

Ginger (*Zingiber officinale* Rosc.), is a herbaceous, rhizomatous, perennial, the underground rhizome of which forms one of the important spices and medicines used by people allover the world. Ginger is marketed in different forms around the world such as raw ginger, dry ginger, bleached ginger, ginger powder, ginger oil, oleoresin, ginger candy, ginger flakes, salted ginger and ginger in brine etc. Gingerale, ginger beer, ginger wine and ginger squash are popular soft drinks. However, dry ginger is the major ginger of commerce. The essential oil from ginger is also used in soaps and perfumery.

Ginger is valued for its medicinal properties and spicy nature. It is widely used in fresh as well as dried forms. The refreshing aroma and pungent taste make ginger an essential ingredient of most world cuisine and of the food processing industry. It has a prime role in primary health care and it has been considered as the 'Mahaoushadi' -the great medicine (Mahindra, 1994). It is a good carminative and digestive. In Chinese and Japanese systems of medicine too, ginger is an important drug.

Though considered to be originated in the South East Asia (Bailey, 1949), at present this spice is cultivated in many tropical countries like India, China, Jamaica, Taiwan, Sierra Leone, Nigeria, Fiji, Mauritius, Indonesia, Brazil, Costa Rica, Ghana, Japan, Malaysia, Bangladesh, Philippines, Sri Lanka, Solomon Islands, Thailand, Trinidad, Tobago, Uganda, Hawaii, Guatemala and many Pacific Ocean islands.

Ginger is mainly cultivated in the tropics from sea level to 1500 m, but it can be grown successfully over more diverse conditions than most other spices. Ginger is thus the most widely cultivated spice (Lawrence, 1984).

The crop prefers a warm humid climate with an annual rain fall of 1500 mm or more. It thrives well on medium loam soils with good supply of humus but it is sensitive to water logging and requires good drainage.

India is the largest producer and exporter of ginger in the world. India produces about 3,07,370 mt of ginger from an area of 8,59,30 ha (2002-03) contributing approximately 30-40% of the world production. About 10 -15% of the total production of ginger is exported to about 50 countries around the world. The crop occupies the largest area in the state of Orissa followed by Karnataka, W. Bengal, Kerala, Meghalaya, and Arunachal Pradesh (Source: Spices Statistics, Spices Board, Kochi, 2004).

Good variability for yield and quality in cultivated ginger is encountered in India and China. The important varieties/cultivars of ginger grown in India are Varada, Rejatha, Mahima, Suprabha, Suruchi, Suravi, Himagiri, Rio-de-Janeiro, Bajpai, Sargiguda, Burdwan, SawThinglaidum, China, Singhihara, Ernad-Chernad, Seirra Leone, Ellakallan, Edappalayam, Himachal, Taiwan, Jorhat, Thang-Chang, Jamaica, Karakkal, Kuruppampady , Thodupuzha, Kunduli Local, Tura, Maran, Uttarpradesh, Valluvanad, Mananthody, Vengara, Nadia, Wyand Local, Narasapattam, Anamika, Assam etc.

Though no seed set is reported in ginger, geographical spread accompanied by genetic differentiation into locally adapted population augmented by mutation could be the main factors responsible for diversity in this clonally propagated crop. The early movement of settlers across the length and breadth of Kerala and the story of shifting cultivation in North Eastern India are well documented sociological events (Sasikumar *et al.*, 1999).

The productivity of ginger is hampered due to the crop loss caused by various pest and diseases. Rhizome rot caused by *Pythium* Spp. (*P. aphanidermatum,* and *P. vexans*), bacterial wilt caused by *Ralstonia solanacearum* (*Pseudomonas solanacearum*) and leaf spot caused by *Phyllosticta zingiberi* are the major diseases (Dake, 1995; Ravindran *et al.*, 2005) whereas, shoot borer (*Conogethes punctiferalis*) and root knot nematode (*Meliodogyne incognita*) are the important insect/nematode pests (Koya *et al.*, 1991; Mohanty *et al.*, 1995; Ramana and Eapen, 1995).

Cultivation of ginger and conservation of genetic resources are threatened by these diseases and pests. None of the existing cultivars are resistant to them. Infected rhizome bits are the primary source of inoculum, as rhizome is the planting unit in ginger. Many a times the rhizomes serve as carriers of dormant pathogenic fungi, bacteria and viruses. Use of disease free planting materials will help to check the spread of diseases.

Biotechnological tools can be used to produce disease free nucleus planting materials and for inducing variability since conventional breeding program is heavily hampered due to lack of seed setting. Use of plant parts other than the rhizome for micropropagation has more relevance in this context. The use of aerial stem (psuedo stem) explant will be an ideal option in this regard, if it can be regenerated directly in enough quantity.

Direct regeneration protocol in ginger is also relevant for genetic transformation, purification of germplasm and in germplasm exchange.

Somatic embryogenesis can be used to induce variability in ginger. This technique can be used directly in somatic hybridization, protoplast culture, synseed production and in genetic transformation (Razdan, 1993).

The present study is an attempt in this direction with the following objectives:

1) Direct *in vitro* regeneration of plantlets from aerial stem (*in vitro* and *in planta* explant).
2) Ontogeny of shoot and root regeneration from aerial stem explant.
3) Regeneration of plantlets from callus of ginger.
4) Induction of somatic embryogenesis from callus cultures of ginger.
5) Ontogeny of somatic embryo induction and shoot / root regeneration from somatic embryos.
6) Micropropagation of plantlets from vegetative buds of ginger.
7) Genetic fidelity studies of *in vitro* derived plants using RAPD markers.
8) Hardening of plants – standardization of hardening medium.
9) Field and quality evaluation of *in vitro* derived plants along with conventionally propagated ginger plants.

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Review of Literature

2. Review of Literature

Ginger (*Zingiber officinale* Rosc.), a rhizomatous monocotyledon, was first described by Rheede (1962) in '*Hortus Indicus Malabaricus*' (Nayar and Ravindran, 1995). Ginger belongs to the family Zingiberaceae, in the order Zingiberales. The family Zingiberaceae consists of 47 genera and about 1400 species (Purseglove *et al.*, 1981). The subfamily Zingiberoideae includes the important spice crops ginger (*Zingiber officinale* Rosc.), turmeric (*Curcuma longa* L.) and cardamom (*Elettaria cardamomum* Maton).

2.1. General

2.1.1. Origin

The country of origin is presumed to be in the region of India or China (Purseglove, 1972). According to Bailey (1949), ginger originated in Western Ghats of India. Though a putative wild type *Zingiber officinale* is collected from Western Ghats of India, its occurrence in wild habitat is yet to be confirmed (Sasikumar *et al.*, 1995).

2.1.2. Botany

Ginger is a herbaceous perennial grown as an annual. The plant is erect, having aerial shoots and underground stem. Aerial shoot (pseudostem) has many leaves borne on very short petioles and with long and narrow sheaths. The leaves are arranged in a distichous manner (Purseglove *et al.*, 1981).

2.1.2.1. Aerial stem

Aerial stem is annual, erect, usually about 50 cm tall, 5 mm in diameter, partly consisting of a pseudostem of overlapping leaf sheaths. The internodal length of aerial stem ranges 3 to 7 cm. (Purseglove *et al.*, 1981).

2.1.2.2. Leaf

The foliage leaf consists of a leaf sheath, a ligule and an elliptical – lanceolate blade. The leaf sheath is about 15 – 18 cm and lamina about 12 – 15 cm long. A distinct mid rib is present in the lamina. Small distichous scale leaves which are brown in color are also present (Purseglove *et al.*, 1981).

2.1.2.3. Roots

Ginger has two types of roots i.e., fibrous and fleshy (Purseglove *et al.*, 1981).

2.1.2.4. Rhizome

The useful part of ginger is the subterranean stem (rhizome) which is modified for the vegetative propagation and storage of food materials. Rhizomes are thick and hard, laterally compressed; often palmately branched. It has nodes with scale leaves and internodes. Each node has an axillary bud except for the first few nodes. The number of nodes in each rhizome varies. The main axis (mother rhizome) and the subsequent branches (primaries) have 6 to 15 nodes. The internodal length of the rhizome branches ranges 0.1 to 1.5 cm. The internodal length is more in secondary and tertiary branches. Each nodes have scale leaves that ensheath and protect the axillary buds (Purseglove, 1972; Ravindran *et al.*, 2005).

2.1.2.5.Inflorescence

Inflorescence arises directly from the rhizome, spiciform 15 – 25 cm long. Single flower is produced in each bract. Flowers are short lived, fragile and each with a bracteole. Fruits are seldom produced (Purseglove, 1972; Ravindran *et al.*, 2005).

2.2. *In vitro* studies

2.2.1. Micropropagation

True-to-type propagation of selected genotypes using *in vitro* culture technique is termed as micropropagation. There are five stages critical for successful micropropagation. They are preparative stage to minimize contamination, initiation of culture, multiplication and elongation of shoots/roots and transfer to green house conditions. The survival, multiplication and field establishment of culture depends upon a variety of factors such as origin of culture, physiological stage of explant, endogenous hormone level and culture environment like nutrient media, photoperiod, CO_2 concentration, temperature etc. Each species is unique in these requirements (De bergh and Read, 1991). Plant regeneration through tissue culture occurs through two methods: organogenesis and embryogenesis.

In ginger, micropropagation studies were carried out by many workers using different explants and media compositions. MS medium (Murashige and Skoog, 1963) was found to be best for micropropagation of ginger. Other basal media used were Gamborg's B5 (Gamborg *et al.*, 1968), Schenk and Hildebrandt's (Schenk and Hildebrandt, 1972) and Norstog's (Norstog, 1973) medium. Mainly used cytokinins were BAP, Kinetin and auxins were, 2, 4-D, NAA and IBA. The different explants used for micropropagation are vegetative buds, inflorescence, single flower and roots.

2.2.1.1. Organogenesis

Organogenesis can be obtained either through direct differentiation of shoot buds from explants (direct organogenesis) or through callus formation in explant and subsequent formation of shoots and roots (indirect organogenesis). Organ formation *in vitro* was reported as early as 1939 when White (1939) observed shoot differentiation in callus cultures of Tobacco.

2.2.1.1.1. Direct organogenesis

Shoots were regenerated directly on explants from numerous species; plants that conventionally propagated adventitiously may be proliferated rapidly *in vitro* by using not only the conventional organ as a source of explants, but other tissues not normally associated with vegetative reproduction (Meins, 1986).

a) Vegetative bud

Vegetative bud is the most commonly used explant in ginger tissue culture. Hosoki and Sagawa (1977) successfully micropropagated ginger from rhizome buds cultured on MS medium supplemented with 2% sucrose and 1 ppm BA. Pillai and Kumar (1982) used SH medium for micropropagation.

Shoot tip explants of ginger cv. Oshouga were planted in both modified Gamborg's B5 medium and Murashige and Skoog (MS) medium containing various levels of NAA and BA. Explants were cultured at 22°C by rotating slowly (2 rpm) under continuous fluorescent light of 2000 lux. Plantlets were produced in modified B5 and MS medium supplemented with 0.2 to 2.0 ppm of BA in addition to 0.2 ppm of NAA. (Sakamura *et al.*, 1986). Bhagyalakshmi and Singh (1988) used MS medium with 6% sucrose, 20% coconut milk, AA at 100 mgl^{-1}, AC at 250 mgl^{-1}, BA at 0.5 mgl^{-1}, IBA at 0.4 mgl^{-1}, and 0.8% agar. A simple and efficient micropropagation method was developed by Inden *et al.* (1988). The medium used was MS with 5 mgl^{-1} BA, 0.5 mgl^{-1} NAA and 20 gl^{-1} sucrose.

Rhizome buds excised from *Curcuma* Spp. and ginger were inoculated on MS medium with different combination of BAP and Kinetin. MS medium with 3.0 mgl^{-1} BA was found to be optimum for *in vitro* clonal multiplication (Balachandran *et al.*, 1990) Choi and Kim (1991) and Choi (1991a) studied the factors affecting *in vitro* response of shoot tip explant. The nutrient medium used was MS supplemented with 0.5 ppm NAA and 5.0 ppm BAP. Multiple shoots were induced from vegetative bud, when cultured on MS medium supplemented with 2.5 mg l^{-1} BA and 0.5 mgl^{-1} NAA

(Dogra *et al.*, 1994). *In vitro* propagation of ginger using vegetative bud cultured on MS medium containing 2 mgl^{-1} BA and 0.6 mgl^{-1}NAA was reported by Huang (1995). Oliver (1996) cultured axillary buds on MS medium supplemented with 8.9 µl BA, 30 gl^{-1} sucrose and 7 gl^{-1} agar and studied the rate of shoot multiplication in each sub culturing.

A protocol for shoot development from disc and apex part of sprouting buds was standardized by Samsudeen (1996). MS medium containing 1µM BAP and 1µM NAA was used for the study. Nirmal Babu (1997) developed a method for clonal propagation of ginger from vegetative bud cultured on MS medium supplemented with 4 mgl^{-1} BA and 1 mgl^{-1} NAA. Disease free plantlets of *Zingiber officinale* were produced by culturing rhizome buds on MS medium supplemented with 3 mgl^{-1} BA and sub cultured to MS with 2 g Twin 40 (a commercial fertilizer medium containing, N, P_2O_5 and K_2O in a ratio of 30:20:10 l^{-1}) with or with out plant growth hormones (Pandey *et al.*, 1997).

High frequency *in vitro* multiplication of ginger was reported by Sharma and Singh (1997). The rhizome buds were cultured on MS medium containing 2 mgl^{-1} Kinetin and 2 mgl^{-1} NAA and 20 g sucrose. A protocol for micropropagation of ginger using vegetative bud was developed by Palai *et al.* (1997). Vegetative buds were cultured on MS medium with 4 - 6 mgl^{-1} BA, 1- 1.65 mgl^{-1} IAA and 100 mgl^{-1} Adenine sulphate and 3 % sucrose. The interaction of growth regulators and culture conditions on micropropagation was also investigated. Freitez and Casares (1997) reported organogenesis from *in vitro* shoot tips cultured on MS medium supplemented with BAP and NAA in different combinations (2, 2.5 & 3 mgl^{-1} BA and 0.25 mgl^{-1} NAA).

A micropropagation protocol for ginger using vegetative bud was standardized by Tyagi *et al.* (1998). The vegetative buds were cultured on MS medium containing BAP and Kinetin in different concentrations. Devi *et al.* (1999)

developed a method for micropropagation from bud explants cultured on MS medium supplemented with BAP and Kinetin (both at 0.1 mgl^{-1}). Shoot production was increased when the culture was transferred to a medium containing BAP at 4 mgl^{-1}. Jasrai *et al.* (2000) propagated ginger plantlets through tissue culture using apical bud on MS medium augmented with different concentration of BAP.

An efficient tissue culture protocol was developed for ginger with a multiplication rate of 1:6 for every 28 days (Rout *et al.*, 2001). Medium containing high level of BAP (3 mgl^{-1} and above) promoted tiny multiple shoots but inhibited root formation. A significant decrease in shoot number per explant was observed at lower (less than 1 mgl^{-1}) concentration of BAP. Ginger plantlets were regenerated from meristem tissues cultured on MS medium with 26.6 µM BA, 8.57 µM IAA and 1111.1 µM adenine sulfate and 3% sucrose. Adaniya and Shirai (2001) reported that the shoot tip explants on soild medium containing 2.0 mgl^{-1} BA, 0.05 mgl^{-1} NAA and 0.2 % (w/v) colchicines for 8 days was most efficient way of inducing tetraploid ginger. The pollen fertility and germinability were also investigated. In the diploid strains, pollen fertility ranged from 0.3 to 6.2% and germination rate from 0 to 0.1% while in the tetraploid strains pollen fertility ranged from 27.4 to 74.2% and the germination rate from 4.8 to 12.9%.

b) *In vitro* aerial stem

In vitro propagation of ginger using *in vitro* aerial stem (pseudostem) explants was reported by Ikeda and Tanabe (1989). Leaf aerial stem and decapitated crown sections from *in vitro* plantlets of *Zingiber officinale* were cultured on MS medium containing various concentrations of BAP and NAA. Pseudostem (aerial stem) cultured on solid medium supplemented with 11 µM BA in combination with 0.6 µM NAA produced an average of 5 shoots and 15.3 roots. Decapitated crown sections cultured in liquid medium with 11 µM BA produced an average of 10 shoots and 16.6 roots.

c) Inflorescence and single flower

There is only one report in ginger regarding the micropropagation using inflorescence. Nirmal Babu *et al.* (1992a) reported direct regeneration of plantlets from immature inflorescence of ginger. One week old inflorescence cultured on MS medium supplemented with 10 mgl^{-1} BA and 0.2 mgl^{-1} 2, 4 – D produced plantlets. Single plantlets were emerged from the ovaries by the seventh week when cultured on MS medium fortified with 10 mgl^{-1} BA and 0.2 mgl^{-1} 2, 4-D.

2.2.1.1.2. Indirect organogenesis

Indirect organogenesis through callus induction and regeneration in ginger was reported by a number of workers. Different explants were used for callus induction. The significant results are summarized below:

a) Vegetative bud / shoot meristem

In ginger, callus induction and regeneration were first reported by Illahi and Jabeen (1987). Shoot bud derived callus on sub culturing onto MS medium supplemented with various concentrations of 2, 4 – D and BA developed plantlets. Malamug *et al.* (1991) induced callus from shoot tip explants of ginger. The medium used was MS with 2, 4-D at 0.5 mgl^{-1} and NAA at 1 mgl^{-1}. These calli produced plantlets when cultured on medium (MS major elements, organic addenda, 2% sucrose and 0.8% agar) with 5 mgl^{-1} BA and 1 mgl^{-1} NAA. Samsudeen (1996) regenerated plantlets from shoot bud derived callus cultured on MS supplemented with 0.1μM 2, 4-D with 50 μM BAP or Kinetin.

Plant regeneration from two callus types in ginger was reported by Ishida and Adachi (1997). These workers studied the varietal differences on callus induction and regeneration. Callus developed by culturing shoot meristem domes of three cultivars of ginger on MS medium containing 2 mgl^{-1} 2, 4-D. 'Taiwan' variety produced compact callus, 'Oshouga' produced sticky callus and 'Indo' produced both compact type and sticky type of calli. Compact callus of 'Taiwan' and 'Indo' varieties

produced only roots in MS with BA. Sticky callus of Indo and 'Oshouga' varieties produced adventitious shoots on MS medium containing 0.1 mgl^{-1} NAA and 1.0 mgl^{-1} BA. These shoots readily formed plantlets on MS medium containing 0.5 mgl^{-1} NAA and 1.0 mgl^{-1} BA. Rout and Das (1997) reported *in vitro* organogenesis in ginger. Callus derived from shoot primordia showed best organogenesis on MS medium supplemented with BA at 5 mgl^{-1}, IAA at 1 mgl^{-1}, adenine sulphate 100 mgl^{-1} and 3% sucrose.

b) Aerial stem

Clonal multiplication of ginger using *in vitro* aerial stem (pseudostem) cuttings was standardized by Choi (1991b). Explants consisted of the base of aerial stem or portion from the top, middle or bottom with one leaf blade attached. All formed callus *in vitro* and from which several plantlets differentiated, although response was best from the base or middle portion explants. Callusing was best on medium containing 0.5 ppm NAA, while shoot and root formation were best on medium containing 0.1-1.0 ppm NAA and1.0 ppm BA.

c) Leaf explant

In vitro plantlet regeneration from leaf derived callus in ginger was reported by Nirmal Babu *et al.* (1992b). Callus induction and proliferation were obtained from leaf tissues cultured on MS medium supplemented with various levels of NAA and 2,4-D. The induced callus produced plantlets when transferred to MS medium containing 0.9 µM 2, 4-D and 44.4 µM BA.

d) Anther

Callus induction from anther tissues of tetraploid ginger was reported by Samsudeen *et al.* (1997). Anthers from diploid and tetraploid plants gave callus on MS medium with 2 mg l^{-1} 2, 4-D. Seven days cold treatment for anther explant and incubated in light were the other conditions required for callus formation. Samsudeen *et al.* (2000) regenerated ginger plantlets from anther derived callus cultures. The media used was MS medium supplemented with 5-10 mgl^{-1} BA and 0.2 mgl^{-1} 2, 4-D.

Investigation of floral structure and plant regeneration through anther culture in ginger was reported by Kim *et al.* (2000). In this study, callus formation on anther explant was compact and embryogenic when cultured on N6 medium supplemented with 2 mgl^{-1} NAA. Plant regeneration occurred on MS medium with BA at 1-2 mgl^{-1}.

2.2.1.2. Somatic embryogenesis

Plant regeneration via somatic embryogenesis in ginger was standardized by Kacker *et al.* (1993). Embryogenic calli were induced by culturing leaf segments taken from *in vitro* shoot cultures on MS medium supplemented with Dicamba 2.7 µM and plantlets were regenerated when the embryogenic cultures were transferred onto MS medium containing 8.9 µM BA. Nirmal Babu *et al.* (1996) regenerated plantlets from somatic embryos of ginger. Ovary explants from 1-2 week old ginger flowers developed profuse callus on MS medium supplemented either with 2,4-D 1 mgl^{-1} or 2,4-D 0.5 mgl^{-1} + BA 1 mgl^{-1}. The callus produced white globular embryoid like structures when cultured on MS medium supplemented with 10 mgl^{-1} BA + 0.2 mgl^{-1} 2,4-D. The embryoids developed into plantlets with better rooting when 1 mgl^{-1} NAA added to the medium. Somatic embryogenesis and its regeneration in ginger were also reported by Tyagi *et al.* (1998). Somatic embryos were induced from leaf derived calli and were regenerated when cultured onto MS with 2.7 µM dicamba and 8.9 µM BAP.

Somatic embryogenic cultures of four ginger cultivars were established by Hua *et al.*, (2005). Somatic embryogenic calli were induced from ginger shoot tips on MS solid medium supplemented with 1.0 mgl^{-1} 2,4-D and 0.2 mgl^{-1} Kinetin which contain only half concentration of NH_4NO_3. Rapid growing and well dispersed suspension cultures were established by sub culturing this callus in the same liquid MS medium. The embryogenic callus was transferred onto solid media (MS + 0.2 mgl^{-1} 2,4-D + 5.0 mgl^{-1} BA + 3% sucrose + 0.7% agar). Somatic embryos produced shoots and roots on MS solid medium with 3.0 mgl^{-1} BA and 0.1 mgl^{-1} NAA. The relationship between the dry weight of suspension cultures and pH changes in

medium is also discussed. A protocol for plant regeneration via somatic embryogenesis of ginger was reported by Suma and Keshavachandran (2005). Callus, derived from young vegetative buds cultured on MS medium supplemented with BA and 2,4-D. Among the plant growth regulators tested, a combination of 2,4-D 1 mgl^{-1} and BA 0.5 mgl^{-1} was most effective in inducing and maintaining embryogenic cultures. The somatic embryos germinated on half strength MS medium with 3% (w/v) sucrose and BA 3 mgl^{-1}.

2.2.2. *Effect of cultural conditions and growth hormones on* in vitro *culture*

In vitro responses of explant to various growth hormones may vary according to the type of explant, plant genotype, cultural conditions and kind of medium. *In vitro* growth and morphogenesis are regulated by the interaction and balance between the growth regulators supplied in the medium and the growth regulators produced endogenously. Auxin and cytokinins are most important for regulating growth and morphogenesis in plant tissue culture (George, 1993a). A balance between an auxin and a cytokinin determine the nature of organogenesis. A high ratio of cytokinin to auxin induced shoot differentiation whereas the reverse favoured root formation; intermediate ratios induced callus formation (Skoog and Miller, 1957).

A study on factors affecting *in vitro* response of shoot tip explants of ginger was conducted by Choi (1991a). The optimal concentration of sucrose in the medium was found to be 3%, and rooting of shoots was enhanced by the addition of 2 gl^{-1} activated charcoal. Rout and Das (1997) studied the effect of media constituents on *in vitro* responses of callus. Organogenesis was best on media supplemented with benzyl adenine (5 mgl^{-1}), IAA (1 mgl^{-1}), adenine sulfate (100 mgl^{-1}) and 3% sucrose. D – Glucose influenced rooting; fructose, maltose and mannitol had no effect on rooting. Shoot bud regeneration was best at pH 5.7 or 5.8 and under 24 h illuminations. The rate of shoot bud regeneration was positively correlated with the concentration of plant growth regulators. Liquid media were less effective than solid media for root

development. Shoots were rooted on half strength MS supplemented with IBA or IAA (1 mgl^{-1}) and 2% sucrose.

The effects of media variables and light conditions on micropropagation were studied by Palai *et al.* (1997). These authors observed that shoot bud multiplication decreased as BA concentration increased from 6 to 8 mgl^{-1}, while IAA had an intermediate effect; NAA, IBA and 2,4-D were ineffective with regard to induction of multiple shoots. Although a high rate of shoot multiplication was noted under both 14 and 24 h of illuminations, shoot elongation was poor under the 14 h photoperiod.

The *in vitro* growth and development of ginger exposed to different levels of NAA (0, 0.25, 0.5, 0.75, 1.0 mgl^{-1}) or BAP (0, 0.5, 1.0, 1.5, 2 mgl^{-1}) and NAA: BAP (0.5:1.0 mgl^{-1}) in solid and liquid media was studied by Arimura *et al.* (2000). NAA increased shoot length in both solid and liquid media. NAA at 0.5 mgl^{-1}promoted the highest number of roots and the longest roots. BAP influenced the number of shoots with maximum response at 1 mgl^{-1}. In liquid media, the absence of BAP induced higher number of shoots. Shoot length was influenced by NAA and BAP. Root number and length were enhanced in liquid media regardless plant growth regulator treatment.

2.2.3. Studies on cost effective media for micropropagation

A simple and cost effective medium for propagation of ginger (*Zingiber officinale* Rosc.) was standardized by Sharma and Singh (1995). Explants from *in vitro* cultured shoots of *Z. officinale* cv. Himachal Local were cultured on MS medium with analytical grade sucrose, ordinary sugar and raw sugar, or on potato extract medium with sucrose, dextrose or sugar. The effects of supplementing the medium with 2 mgl^{-1} BA, omitting agar and replacing distilled water with tap water in the preparation of the medium were also investigated. The highest number of shoots/ explant was produced on MS medium + ordinary sugar and no growth regulators. Use of tap water and omission of agar also gave good results. These techniques can used

to reduce the cost of micropropagation. Economics of mass *in vitro* propagation of vanilla and ginger was studied by Rao *et al.* (1998).The cost of production of tissue cultured plantlets of ginger and vanilla was determined, based on 14168 plantlets of the former and 3560 of the latter, produced in the tissue culture laboratory during 1994 – 95. Production costs were Rs. 2.77/plantlet for ginger and Rs. 5.64/plantlet for vanilla. Ginger had a higher multiplication rate and required shorter subculture intervals than vanilla.

2.2.4. Screening of micropropagated ginger plants for disease resistance.

Selection of *Pythium* tolerant ginger by subjecting cell cultures to *Pythium* culture filtrate and subsequently regenerating plants from the surviving cells was attempted by Kulkarni *et al.* (1984). Regenerated plants were again screened for tolerance to *Pythium* which were enabled to isolate three lines tolerant to *Pythium.*

Virus diseases of ginger were controlled in tissue culture by heating to 50°C for 5 minutes. Cultures were grown in MS medium with 2 mgl^{-1} BA + 0.2 mgl^{-1} IAA, then in the same medium with half the concentrations of BA and IAA, followed by the final transfer to a B5 medium with 0.2 mgl^{-1} IAA. The cultivation of virus free stock to give high yields of 5 t /667 m^2 is discussed (Gao *et al.*, 1999).

2.3. Histological studies

2.3.1. Tissue cultured plant materials

Histological origin of *in vitro* regenerated organs of ginger (*Zingiber officinale* Rosc.) was described by Freitez *et al.* (1998). Samples were taken during the initiation and multiplication phases of *Z. officinale* cultures. During the initial phase, progress was observed in the development of shoot tips in terms of the number and size of cells and foliar and axillary bud development. Likewise at 21 days the endogenous differentiation of a root primordium or meristemoid was observed,

starting from the cells of the procambium with their respective vascular connections. At 28 days, there was a great proliferation of vascular bundles oriented towards the adventitious axillary buds, the latter originating from *de novo* tissue. In the multiplication phase a great profusion of buds and adventitious roots without any type of polarity was observed, similar to a rosette; finally achieving a high *in vitro* multiplication rate. Histological analysis confirmed that the development of buds as well as of roots occurred through organogenesis without passing through callus tissue, through the process of induction and differentiation of the tissues. In spite of their adventitious nature, the plants obtained were morphologically and genetically uniform.

Histological examination of embryogenic calli was done by Kacker *et al.* (1993). The study revealed the presence of nodular structures containing richly cytoplasmic cells delimited by a single layered epidermis. The first sign of embryogenesis was marked by the appearance of white globular structures that were attached to the surface of nodular callus by a distinct stalk. Ontogeny of shoot and roots from ginger callus was described by Nirmal Babu (1997).

2.3.2. Aerial stem

No report is available on the anatomical studies of aerial stem in ginger. Tomlinson (1969) described the internal structure of aerial stem of Zingiberaceous plants in general. In the internodal region cortex and central cylinder are always distinct, usually separated from each other by a narrow fibrous cylinder; cortex fairly wide, including one or more rings of vascular bundles; central cylinder including many scattered vascular bundles. In the node region main leaf traces entering stem gradually and not forming a vascular plexus. Axillary bud present in each node.

2.3.3. Leaf

Ginger leaves are isobilateral. The upper epidermal cells of leaf are polygonal. In the lower epidermis the cells are polygonal and irregular. Oil cells are present in

the epidermis. Unicellular hairs are present in the lower epidermis of the foliar leaves. Ginger leaves are amphistomatic. A distinct substomatal chamber is present. Tomlinson (1969) gave a brief note on the petiolar anatomy of ginger. The short petiole shows a swollen pulvinus like appearance. Two bundles arcs, air canals and assimilating tissues are absent in the pulvinus. Collenchymatous thickening of the cells of bundle sheath is present in the pulvinus.

Comparative foliar anatomy of ginger plants under three different growing conditions was reported by Freitez and Casares (2004). *In vitro*, acclimatization and field experiments were conducted to describe the foliar anatomy of ginger that may influence their establishment. Samples were prepared from transverse sections of the foliar lamina. There were anatomical difference in stomata location, cuticle thickness and vascular sheath location and shape.

2.3.4. Rhizome

The developmental pattern, growth and branching behaviour of rhizomes of ginger were studied. Axillary buds are present at each node. Secondary and tertiary branches originate from the axillary bud from the adaxial sides of the leaf or scale leaf (Remashree *et al.*, 1998).The T.S of rhizome has a distinct layer of epidermis consisting of a single raw of rectangular cells. Within this is the cork composed of irregularly arranged, tangentially elongated, slightly brown colored cells and an inner zone of 6 to 12 regular rows of thin walled rectangular to slightly tangential elongated cells arranged in radial rows. Inner to the cork is the cortex, composed of thin – walled large hexagonal to polygonal parenchymatous cells. The cortical region is heavily loaded with starch grains and oil cells. Many scattered, collateral, closed vascular bundles are present, within which are mostly concentrated in the inner cortical zone. The inner limit of the cortex is marked by a single – layered endodermis composed of thin – walled rectangular cells. Inner to this is the stele consists of parenchymatous cells. It also contains starch grains, oil cells and vascular bundles (Ravindran *et al.*, 2005).

In ginger, rhizome enlargement is due to the activity of three meristamatic zones (primary thickening meristem, actively dividing parenchyma and secondary thickening meristem). The scattered vascular bundles are developed from the primary thickening meristem (PTM) or procambial cells. Unlike in many monocots, in ginger there is a special meristamatic layer along with the endodermoidal layer and this layer consists of cambium like cells. The presence of cambium like cells is an important feature in rhizome development. From this layer, inverted and irregularly distributed groups of xylem and phloem are formed along the intermediate layer. The cells outer and inner to the cambial layer become filled with starch grains (Remashree *et al.*, 1997; Ravindran *et al.*, 2005). Minute glands containing essential oil and resin are scattered throughout the rhizomes, but numerous in the epidermal tissue (Guenther, 1952; Mangalakumari *et al.*, 1984).

2.3.5 Embryology of angiosperms

Embryogeny: The zygote divides transversely, resulting into a small apical cell toward the interior of the embryosac and a large basal cell towards the micropyle. Then these cells followed 4 celled and 8 celled stages. It is at the octant stage of the proembryo that the destinies of various cells become determined. Wardlaw (1955) remarked that in monocotyledonous embryo the shoot apex occupies a lateral position in the somewhat cylindrical embryo and the cotyledon is terminal. A mature embryo of *Triticum* has a single cotyledon called scutellum. In median longitudinal section of the mature embryo it appears laterally attached to the embryonal axis. The portion of the embryonal axis below the level of scutellum is the radicle, which bears an apical meristem and a root cap at the lower end. The radicle and its cap are enclosed in coleorhiza, which is the undifferentiated lower part of the proembryo. The portion of the embryonal axis above the level of scutellum is the epicotyl. It comprises a shoot apex with some leaf primordial, enclosed in a hollow foliar structure, the coleoptile (Batygina, 1969).

2.4. Genetic fidelity analysis in ginger

Genetic fidelity of micropropagated plants are always a concern for horticulturists. RAPD (Random Amplified Polymorphic DNA) markers were used to evaluate the genetic stability of micropropagated plants of *Zingiber officinale* Rosc. (Rout *et al.*, 1998). These authors reported that RAPD profiles did not indicate any polymorphism among the micropropagated plants. Suja (2002) and Nirmal Babu *et al.* (2003) used RAPD profiles amplified by 11 Operon primers as an index for estimating genetic fidelity of selected 'variants' among micropropagated and callus regenerated ginger plants. They observed differences in RAPD profiles of some micropropagated plants. It indicated that micropropagation even without any callus phase induced variations.

2.5. Hardening

The physiological and anatomical characteristics of micropropagated plantlets necessitate that they should be gradually acclimatized to the *ex vitro* conditions to prevent high mortality after transfer to the environment of the field (Hazarika, 2003).

For acclimatizing the micropropagated ginger plantlets, a number of hardening media have been used. Plant acclimatization was completed with laboratory hardening under 85 $\mu E\ m^{-1}\ s^{-1}$ light for 7 days and then plants were transplanted into substrates in humidified chambers in the shade (Freitez and Casares, 1997). Hardened plants could establish in the field with a survival rate of 80-98% (Roy and Wamanan, 1990; Samsudeen, 1996; Nirmal Babu, 1997; Sharma and Singh, 1997; Palai *et al.*, 1997). Jasrai *et al.* (2000) could established tissue cultured ginger plantlets in media containing garden soil, sand and compost in the ratio of 40:20:40 and got 98% survival.

2.5.1. Biological hardening

Four antagonistic bacterial isolates, *Bacillus subtilis*, *Bacillus* sp., *Pseudomonas corrugata* 1 and *P. corrugata* 2, isolated from the rhizosphere of tea plants growing in different geographical locations in India, were tested as microbial inoculants for hardening of tissue-cultured tea plants raised in the laboratory prior to the transfer to open land. Bacterial inoculations resulted in enhanced survival (up to 100, 96, and 88%), as against 50, 52, and 36% survival observed in the corresponding control plants, in rainy, winter and summer seasons, respectively. Rhizoplane and rhizosphere soil analyses showed that the major biotic factor responsible for mortality following the transfer of tissue culture raised plants to soil was fungal attack (*Fusarium oxysporum*). Bacterial inoculations also resulted in plant growth promotion of tissue culture as well as seed raised plants of tea (Pandey *et al.*, 2000).

Piriformospora indica is a novel plant growth promoting root endophyte. Regenerated plantlets of tobacco subjected to two different biological hardening techniques showed 88-94% survival when inoculated with *P. indica* as compared to 62% survival of un inoculated controls under similar conditions. The tendency of the plantlet to overcome the stress in terms of maximum revival capacity was in the case of *P. indica* as compared to the control. The fungus has the potential to render protection to the micropropagated plantlets and help them escape the 'transient transplant shock' (Sahay and Varma, 1999).

Shailesh and Kothari (2006) reported that soil: press mud cake (3:1) is cost-effective and eco-friendly potting medium for hardening of *Musa paradisiaca* L. Incorporation of *Azotobacter, Aspergillus* and VAMs during media preparation helps in better establishment and growth of plants.

2.6. Field evaluation

2.6.1. Morphological and yield characterization in micropropagated plants.

Field evaluation of micropropagated and conventionally propagated ginger in subtropical Queensland was reported by Smith and Hamil (1996). In the first generation *ex vitro*, micropropagated ginger plants had significantly reduced rhizome yields with smaller knobs and more roots as compared to the control plants. Micropropagted plants had a grater shoot: rhizome ratio. Shoots from micropropagated plants were also significantly smaller, with a grater number of shoots per plant. Rhizomes collected from micropropagated plants were smaller than that of control plants. By the second generation *ex vitro*, there was no significant difference in any of the parameters measured between the treatments.

The morphological and yield characteristics of *in vitro* derived plants; micropropagated plants derived from vegetative buds (MP), callus regenerated plants (C) along with the conventionally propagated ones (CP) were studied by Samsudeen (1996). The characteristics observed are number of tillers – 1.40 to 10.00 (MP), 1.20 to 8.30 (C), 2.80 to 9.20 (CP); number of leaves - 11.40 to 57.60 (MP), 8.30 to 64.90 (C), 27.60 to 69.10 (CP); height of the plant- 8.40 to 36.90 cm (MP), 7.60 to 35.80 cm (C), 15.50 to 36.70 cm (CP) and rhizome weight – 10.10 to 57.80 g (MP), 8.40 to 34.80 g (C), 15.10 to 41.50 g (CP). Sharma and Singh (1997) reported the field performance of *in vitro* derived plants. The micropropagated plants were morphologically identical to the mother plants and were free of ginger yellows disease. They performed well under field condition and well developed rhizomes obtained from tissue cultured plants did not rot during storage for up to 6 months.

Morphological characterization of 4 year old micropropagated as well as callus regenerated plants was reported by Nirmal Babu (1997) in comparison with conventionally propagated plants. Direct regenerated plants and callus regenerated plants as separate groups when compared with conventionally propagated plants

revealed a good amount of variation with regard to plant height, number of nodes per finger and leaves per plant, girth of rhizome, number of nodes per finger, internodal distance and yield per plant. Micropropagated and callus regenerated plants have higher mean values with regard to plant height, number of tillers, number of nodes per finger and yield per plant compared to controls. With regard to the width of rhizome, internodal distance all three groups are at par.

Morphological features and yield of tissue culture raised ginger plants were studied by Rao *et al.* (2000). The features observed were; number of leaves/tiller (10.00 – 13.00), length of sixth leaf from top (22.00 – 23.00 cm), breadth of sixth leaf from top (2.20 - 2.30 cm), plant height (45.00 - 55.00 cm), number of tillers/plant (14.00 – 19.00) and fresh weight of rhizome/plant (55.00 – 66.00gm). The vegetative growth was normal and the yield was more or less comparable with that of conventionally propagated ginger plants. It took four months and fifteen days extra to harvest the rhizome in case of tissue cultured plants.

Field evaluation of ginger plants (*Zingiber officinale* Rosc.) obtained *in vitro* and from sections of rhizome was reported by Freitez *et al.* (2003). The horticultural performance of ginger (*Z. officinale* Rosc.) plants cultured *in vitro* and propagated from rhizome sections was evaluated under full sunlight and partial shade in Tarabana, Lara State, Venezuela. The results showed significant differences for the type of propagation and the condition of light, except for the variables, number of leaves and root mass, respectively. The number of shoots, and fresh and dry mass of shoots was higher in *in vitro* propagated plants, which were shorter, than those propagated by rhizome. Rhizome mass was greater in plants propagated conventionally, but root mass was smaller than in those propagated *in vitro*. The *in vitro* plants produced numerous small rhizomes, with a high number of fleshy roots and tuberous structures at the tips. All the evaluated variables were superior in partial shade, independently of the type of propagation, with the exception of roots mass in those plants produced from rhizome sections.

2.6.2. Morphological and yield characterization in conventionally propagated ginger

Field evaluation of ginger plants was reported by a number of workers. Rattan (1989) indicated that number of leaves per plant had maximum direct contribution to yield per plant, followed by rhizome breadth.

Multiple regression analysis by using morphological characters indicated that the final yield could be predicted fairly accurate by taking into consideration plant height, number of leaves and breadth of last fully opened leaf at the 90^{th} and 120^{th} days after planting (Rathnambal *et al.*, 1980). Morphological variability in ginger was reported by a number of workers (Table 1).

Table 1. Morphological variability in ginger

Sl. No.	Character	Range	References
1	Plant height (cm)	35.41 – 49.67	Muraleedharan and Sakunthala, 1975.
		49.90 – 64.00	Saikia and Shadeque, 1992.
		23.13 – 88.60	Sasikumar *et al.*, 1992; Ravindran *et al.*, 1994.
2	Tillers/plant	5.33 – 11.91	Muraleedharan and Sakunthala, 1975.
		4.00 – 6.80	Saikia and Shadeque, 1992.
		2.75 – 37.50	Sasikumar *et al.*, 1992; Ravindran *et al.*, 1994.
3	Leaf number/plant	56.66 – 146. 75	Muraleedharan and Sakunthala, 1975.
		20.00 – 58.00	Saikia and Shadeque, 1992.
		17.00 – 52.00	Sasikumar *et al.*, 1992; Ravindran *et al.*, 1994.
4	Rhizome yield/plant	116.00 – 286.96	Muraleedharan and Sakunthala, 1975.
		25.00 – 57.00	Saikia and Shadeque, 1992.
		55.00 – 770.00	Sasikumar *et al.*, 1992; Ravindran *et al.*, 1994.

2.6.3. Effect of planting season in growth and yield of ginger

Ginger needs a warm climate and cannot withstand cold and hence its planting should be adjusted to avoid the winter cold. In India ginger is planted with commencement of South west Monsoon. It generally takes place in May to June (Khan, 1959). Planting time is crucial in ensuring high yield. Too early planting while soil temperature is low will affect germination. If planted too late, the growing period will be shorter and the rhizome yield will be affected. Studies have shown that delay in planting results in a lower yield of ginger. As vegetatively propagated plants, the whole growing period is utilized for vegetative growth only. Growth period takes more than 135 – 150 days from sprouting. Harvesting of ginger is generally done between January and February (Govindarajan, 1982).

2.6.4. Effect of shading in growth and yield of ginger

Ginger belongs to the medium light plant and endures high temperature. If shading or other measures to lower the temperature and hold humidity are not implemented, ginger seedlings will be weak and short, resulting in yield declines. Shading can make ginger leaves sustain a higher photosynthetic efficiency and high dry matter accumulation, there by increasing the unit area yield (Zhenxian *et al.*, 2000). 50 – 60 shading is suitable for better growth of ginger (Shaottui and Zhenxian, 1998, Xizhen *et al.*, 2001).

2.7. Correlation and path analysis

Mohanty and Sarma (1979) studied the genetic variability and correlation for yield and other variables in ginger germplasm and reported significant positive correlations between rhizome yield and various yield attributes such as plant height, leaf and tiller number, length and width of leaves.

One hundred collections of ginger evaluated for plant height, leaf number, tiller number, leaf length and width, days to maturity, dry recovery as well as rhizome yield/plant revealed good variability for tiller number and rhizome yield/plant.

Moderate variation was observed for plant height, leaf number, tiller number as well as length and width of leaves. These characters had positive significant association with rhizome yield. Plant height, followed by leaf length, had maximum direct effects on rhizome yield (Sasikumar *et al.*, 1992).

Das *et al.* (1999) reported very high positive direct effect of stomatal number, leaf area, leaf number and plant height on rhizome yield.

Singh (2001) evaluated sixteen clones of ginger for certain metric traits in a field experiment in Himachal Pradesh, India, during the Kharif season of 1996-97. Correlation studies revealed that rhizome yield per plant was positively and significantly correlated with plant height, number of leaves per plant, number of tillers per plant, leaf length and leaf width. The maximum indirect effects on yield were observed for number of leaves through plant height, followed by leaf length through plant height.

The number of tillers, tiller height, leaflet number, leaflet length, leaflet breadth, rhizome length and width, thickness of basal pseudostem, thickness of primary, secondary and tertiary rhizomes and fresh weight of rhizomes recorded from 40 ginger genotypes were studied by correlation and path analysis (Abraham and Latha, 2003). There was a high positive association between yield and leaflet number followed by tiller height, rhizome length, leaf length and thickness of the secondary rhizome. Path analysis revealed that characters such as leaflet number, rhizome length, thickness of the secondary rhizome, rhizome width and leaflet length have high positive direct effect on yield.

2.8. Quality evaluation

Ginger rhizome contains volatile oil, fixed oil, pungent compounds, starch and other saccharides, proteins, crude fiber, waxes, coloring matter and trace minerals. The presence of vitamins and amino acids also has been reported. The relative

percentage of these components varies with cultivar, soil and climatic differences. Starch is the most abundant of the constituents, comprising of 40 – 60 % of the weight of the dry rhizome (Lawrence, 1984).

Fresh ginger contains 80.9% moisture, 2.3% protein, 0.9% fat, 1.2% minerals, 2.4% fibre and 12.3% carbohydrates. The minerals present in ginger are iron, calcium and phosphorous. It also contains vitamins such as thiamine, riboflavin, niacin and vitamin C. The powdered rhizome contains 3 – 6% fatty oil, 9% protein, 60 – 70% carbohydrates, 3 – 8% crude fibre, about 8% ash, 9-12% water and 2-3% oil (Kalpagam and Nirmala, 2003).

By steam distillation or extraction with supercritical carbonic acid dioxide, ginger rhizome gives an essential oil with a high content of mono and sesqui terpene derivatives (α – zingiberene). By extraction with solvents, an oleoresin containing the pungent principles of ginger is obtained.

2.8.1. Ginger oil

Dry ginger contains 1.25 to 3.00 % essential oil (volatile oil), which imparts the characteristic aroma to the spice. The oil is devoid of the pungent taste of ginger. Salzer (1977) and Sankarikutty *et al.* (1980) suggested the following determinants of quality for ginger oil.

a) Citral and citronillyl acetate are important co determinants of odor.
b) Zingiberene and beta – sesquiphellandrene are the main components of the freshly prepared oil. These components are converted to ar – curcumine with storage.
c) The ratio of zingiberene + beta sesquiphellandrene to ar – curcumine is indicative of the age of the oil.

Oil composition of fresh and dried ginger rhizome of Nigeria was investigated by means of a combination of column chromatography, high resolution GC and GC – MS. The essential oil contained mainly mono and sesquiterpenoids of which Geraniol, Neral, 1, 8 - Cineol, Zingiberene, β – Sesquiphellandrene were the major components (Ekundayo *et al.*, 1988).

2.8.2. Ginger oleoresin

The oleoresin, obtained by extraction of the spice with volatile solvents, contains the aroma as well as the taste principles of the ginger in highly concentrated form. Oleoresin represents the wholesome flavor of the spice – a cumulative of the sensation of smell and taste. It consists of the volatile essential oil and nonvolatile resinous fraction comprising taste components, fixatives, antioxidants, pigments and fixed oils naturally present in the spice. The oleoresin is therefore, designated as "true essence" of the spice.

The pungency of ginger is contributed by gingerols (Nambudiri *et al.*, 1975). The oleoresin of ginger contains gingerols as well as degenerated products. Other pungent components in ginger oleoresin are shogoal, paradol, zingereone, gingediol, gingerdione and gingediacetae. On long term storage gingerol become converted to shogoal.

2.8.3. Biochemical variability in micropropagated plants

Rhizomes formed *in vitro* from shoot cultures of ginger were analyzed for flavor and pungency compounds. Essential oil from *in vitro* developed rhizome contained same constituents as in the original rhizomes, but quantitative difference was observed. Oil composition also depended on the composition of the basal medium. Oil extracted from the rhizome grown in the modified B5 medium, the acyclic oxygenated monoterpenes predominated, while the oil from the rhizome grown in modified MS medium consisted mainly of sesquiterpenes. (Sakamura *et al.*, 1986; Charlwood *et al.*, 1988; Sakamura and Suga, 1989).

Quality analysis of rhizomes obtained from micropropagated plants was done by Bhagyalakshmi and Singh (1988). They reported micropropagated plants were at par with conventionally propagated ones except that they need longer (additional 2 months) crop duration for the same effect. In another study, Bhagyalakshmi *et al.* (1994) studied the periodic fluctuations in the yield and rhizome composition of various compounds in micropropagated ginger plants. Conventionally propagated ginger is usually harvested after 8 months and micropropagated plants harvested at the same time were comparable qualitatively and quantitatively to the control rhizomes in the composition of starch, ash, acetone extract and volatile extract but only qualitatively similar in total oleoresin content, flavor profile, TLC aromagram and GC analysis, with less of each component than control rhizomes.

Biochemical parameters of ginger somaclones were studied by Samsudeen (1996). The somaclones had significant exploitable variations with regard to biochemical characters like, content of oleoresin, dry recovery and fiber content. In the case of micropropagated plants, oleoresin content of 3.3 - 9.3 %, fibre 3.7 - 6.7% and dry recovery 12.1 - 36.4% were recorded. In callus regenerated plants an oleoresin content of 3.4 - 9.4%, fibre 1.3 - 5.3% and dry recovery of 14.5 - 37.5 % were recorded as compared to control plants (oleoresin content –3.9 - 5.0%; fibre – 4.3 - 5.5%; dry recovery – 19.6 - 26.4%). Nirmal Babu (1997) corroborates this result. Variations were observed among tissue cultured and callus regenerated plants in their oleoresin content, fiber content and dry recovery. In the case of micropropagated plants (oleoresin 2.4 -7.6%; fibre 3.8 – 6.7%; dry recovery 21.2 – 31.8%) and in the callus regenerated plants (oleoresin 1.6 – 4.5%; fibre – 3.6 – 6.0%; dry recovery – 21.9 – 29.4%) whereas in the control plants (oleoresin 5.20%; fibre 5.90%; dry recovery 28%). Qualitative studies on *in vitro* derived ginger plants were carried out by Rao *et al.* (2000). The oleoresin (11.34%) and oil contents (3.60%) in the exotic material from Jamaica were found to be superior to the local ginger varieties viz. Kurupampady (oleoresin – 7.44% and oil content – 2.13%) and Cochin ginger (oleoresin – 7.43% and oil content 2.38%).

2.8.4. Biochemical evaluation in conventionally propagated ginger

Biochemical properties of ginger were studied by various workers. The details are given in the Table 2.

Table 2. Quality/biochemical parameters of ginger rhizome.

Sl. No.	Character	Range	References
1	Dry recovery	17.70 – 25.09 %	Muraleedharan and Sakunthala, 1975.
		14.00 – 28.00 %	Sasikumar *et al.*, 1992; Ravindran *et al.*, 1994.
2	Volatile oil	1.25 – 2.81 %	Natarajan *et al.*, 1970
		1.00 – 2.70 %	Natarajan *et al.*, 1972
		1.13 – 2.56 %	Saikia and Shadeque, 1992
		1.80 %	Peter and Kandiannan, 1999
3	Oleoresin	3.72 – 7.10 %	Muraleedharan and Sakunthala, 1975.
		4.90 – 10.50 %	Nybe *et al.*, 1980.
		3.00 – 10.80 %	Sreekumar *et al.*, 1980.
		3.20 – 9.50 %	Sasikumar *et al.*, 1992; Ravindran *et al.*, 1994.
4	Crude fiber	1.40 – 9.50 %	Natarajan *et al.*, 1970
		4.80 – 9.80 %	Natarajan *et al.*, 1972
		4.23 – 8.13 %	Muraleedharan and Sakunthala, 1975.
		3.40 – 6.40 %	Nybe *et al.*, 1980.
		3.50 – 6.00 %	Sreekumar *et al.*, 1980.
		4.56 – 8.05 %	Saikia and Shadeque, 1992
		2.10 – 7.00	Sasikumar *et al.*, 1992; Ravindran *et al.*, 1994.
		7.17 %	Peter and Kandiannan, 1999
5	Carbohydrate	60.00 – 70.00 %	Kalpagam and Nirmala, 2003.
6	Starch	41.54 – 55.06 %	Natarajan *et al.*, 1970
		40.40 – 59.00 %	Natarajan *et al.*, 1972
		39.80 – 53.00 %	Saikia and Shadeque, 1992
		53.00 %	Peter and Kandiannan, 1999

INVESTIGATION ON DIRECT *IN VITRO* SHOOT REGENERATION FROM AERIAL STEM EXPLANT OF GINGER (*Zingiber officinale* Rosc.) AND ITS FIELD EVALUATION

Thesis submitted to the

University of Calicut

For the award of degree

of

Doctor of Philosophy

(BOTANY)

by

Lincy A K

University of Calicut

Calicut, Kerala, India

2007

Materials and Methods

3. Materials and Methods

The present study was conducted at Indian Institute of Spices Research (IISR), Calicut, Kerala during the year 2002-03 through 2005-06. The materials and methods used in this study are given below:

3.1. *In vitro* studies

3.1.1. Materials

3.1.1.1. Source of explants

Ginger (*Zingiber officinale* Rosc.) var. Jamaica (an exotic type) and var. Varada (one of the most important ruling varieties in India) were used in the study. Rhizomes of these varieties were collected from the ginger gene bank of IISR, Calicut. The details of the different explants used in the study are given below:

a) Direct regeneration

- Aerial stem (*in planta* and *in vitro*).
- Vegetative buds.

b) Callus induction

- Aerial stem (*in planta* and *in vitro*).

c) Somatic embryogenesis

- Callus derived from aerial stem (indirect somatic embryogenesis).
- Aerial stem (*in planta*) explants.
- Leaf (*in planta* and *in vitro*).

3.1.1.2. Culture medium

MS medium (Murashige and Skoog, 1963), the most commonly used medium for plant tissue culture was used in the present study. Composition of MS basal media is given in Table 3.

Table 3. Composition of Murashige and Skoog basal medium.

Composition	Chemical formula	Concentration (mgl^{-1})
Macro nutrients		
Ammonium nitrate	NH_4NO_3	1650.00
Potassium nitrate	KNO_3	1900.00
Calcium chloride	$CaCl_2.2H_20$	440.00
Potassium orthophosphate	KH_2PO_4	170.00
Magnesium sulphate	$MgSO_4.7H_2O$	370.00
Micro nutrients		
Sodium EDTA	Na_2EDTA	37.30
Ferrous sulphate	$FeSO_4.7H_2O$	27.80
Boric acid	H_3BO_3	6.20
Manganese sulphate	$MnSO_4.4H_2O$	22.30
Potassium iodide	KI	0.83
Zinc sulphate	$ZnSO_4.7H_2O$	8.60
Sodium molybdate	$Na_2MoO_4.2H_2O$	0.25
Copper sulphate	$CuSO_4.5H_2O$	0.025
Cobalt chloride	$CoCl_2.6H_2O$	0.025
Vitamins		
Myo inositol	$C_6H_{12}O_6$	100.00
Thiamine HCL	$C_{12}H_{17}CIN_4OS.HCl$	0.10
Nicotinic acid	$C_6H_5NO_2$	0.50
Pyridoxine HCL	$C_8H_{11}NO_3.HCL$	0.50
Amino acid		
Glycine	$C_2H_5NO_2$	2.00

The chemicals used for macro and micro nutrients were obtained from 'Hi-Media', Mumbai. Sucrose (Hi-media, Mumbai) was used as the carbon source at the rate of 30 gml^{-1}in all the experiments.

3.1.1.2.1. Growth regulators

The growth regulators used in the present study are:

a) Auxins: Three major auxins, namely α – Naphthalene acetic acid (NAA), Indole, 3-butyric acid (IBA) and 2, 4-dichlorophenoxy acetic acid (2, 4-D).

b) Cytokinins: Three cytokinins namely, 6-Benzyl amino purine (BAP), 6-furfuryl amino purine (Kinetin) and Thidiazuron (TDZ) or (N-Phenyl-N-1, 2, 3-thidiazol-5' – yl-urea).

c) Gibberellic acid and Abscisic acid.

3.1.1.2.2. Gelling agent

For solidifying the culture medium, bacteriological grade Agar agar (Hi-media, Mumbai) was used at the concentration of 7 g l^{-1}

3.1.1.3. Distilled water

Double glass distilled water was used for rinsing the glassware and preparation of stocks, growth regulators, media and buffer etc.

3.1.1.4. Glass wares

For callus induction, callus multiplication and somatic embryo induction, Borosil Petri plates were used. Borosil culture tubes (15 x 200 mm) and screw cap bottles (30 ml) were used for direct regeneration, callus regeneration and somatic embryos regeneration. For shoot multiplication, Borosil conical flasks (250 ml) were used.

3.1.1.5. Plugging the culture vessels

The culture tubes and flasks were plugged with cotton plugs made of non-absorbent cotton covered with gause.

3.1.1.6. Sterilization of culture medium

The medium was sterilized by autoclaving at 120°C for 15 minutes at 1.06 Kg cm^{-2} pressure.

3.1.1.7. Instruments

Inoculation under aseptic conditions was done in 'Thermadyne' horizontal laminar airflow chamber. 'Nat steel' horizontal autoclave was used for sterilizing the culture media and other materials like blades, blade holders, forceps, cotton, distilled water, and filter pepper etc.

For accurately weighing the chemicals, agar, sucrose, hormones etc. 'Mettler Toledo' weighing balance was used. For pH analysis 'Elico India' pH meter was used.

3.1.1.8. Incubation condition

The cultures were maintained in an air conditioned room at 22±2°C and relative humidity around 60%, photoperiod regime of 16 hr light and 8 hr dark with a light intensity of 3000 lux, provided by 'Philips' cool white fluorescent tubes.

3.1.2. Methods

3.1.2.1. Preparation of nutrient medium

Stock solutions were prepared for macro nutrients, micro nutrients, iron salts, Myo-inositol, amino acids and vitamins. The required quantity of chemicals were weighed and dissolved in double distilled water (Table 4).

Table 4. Stock solutions for MS medium.

Stock	Chemical formula	Concentration (g)	Stock strength	Quantity for 1 liter medium.
Stock A				
Macro	NH_4NO_3	33.00		
nutrients	KNO_3	38.00		
(1000 ml.)	$MgSO_4.7H_2O$	7.40		
	KH_2PO_4	3.40	X 20	50 ml
	* $CaCl_2.2H_2O$	6.60		
	H_3BO_3	0.310		
	$MnSO_4.H_2O$	1.115		
Stock B				
Micro	KI	0.042		
nutrients	$ZnSO_4.7H_2O$	0.430	X 100	20 ml
(500 ml).	Na_2MoO_4	0.013		
	* $CuSO_4.5H_2O$	0.00125		
	* $CoCl_2$	0.00125		
Stock C				
Micro	* $FeSO_4$	1.390	X 100	20 ml
nutrients.	* Na_2EDTA	1.865		
(500 ml.)				
Stock D				
Vitamins.	$C_6H_5NO_2$	0.025		
(500 ml.)	$C_8H_{11}NO_3.HCl.$	0.025	X 100	20 ml
	$C_{12}H_{17}ClN_4O_5.HCl$	0.005		
	$C_2H_5NO_2$	0.1		
Stock E				
Inositol	$C_6H_{12}O_6$	5.0	X 100	20 ml
(500ml.)				

* Dissolve separately before adding to the final stock solution.

Separate stock solutions for each hormone were also prepared in sterile double distilled water. All stock solutions were stored in refrigerator (4°C).

To prepare the nutrient media, the required amount of stock solutions (macro nutrients, micro nutrients, vitamins, amino acids and growth hormones) sucrose (30 gl^{-1}) and growth hormones were taken and final volume was made up by adding sterile double distilled water. The pH of the medium was adjusted to 5.8 using 0.1 N. NaOH or HCl. For solidifying the medium, agar agar was used at 0.7% (w/v). After melting the agar, the media were dispensed into appropriate containers and sterilized

by autoclaving. The thermoliabile hormones such as GA_3 and abscisic acid were sterilized with 'Millipore' filter sterilization system with 0.22 μ pore size and dispensed to sterilized nutrient media under laminar air flow chamber. MS half strength media was used for all experiments.

3.1.2.2. Aseptic inoculation

Prior to use, the Laminar air flow chamber was wiped with 70% alcohol and sterilized under UV light for 30 minutes. All the surgical instruments were autoclaved, dipped in alcohol, flamed and cooled before use.

3.1.2.2.1. Surface sterilization of in planta *explants*

Explants collected from disease free plants / rhizomes were thoroughly washed in tap water and disinfected by washing with 5% detergent solution (Teepol) for 20 minutes followed by 2-3 washes in sterile distilled water. The explants were cut into convenient size. Further steps were carried out under laminar air flow chamber.

The explants were surface sterilized with 0.1% $HgCl_2$. The time duration of mercuric chloride treatment varied with the type of explants. Leaf tissues were treated with 0.1 % $HgCl_2$ for 5-7 minutes, aerial stem cuttings and vegetative buds were surface sterilized with 0.1% $HgCl_2$ for 10 minutes. After the $HgCl_2$ treatment, the explants were rinsed 4-5 times with sterilized double distilled water to ensure complete removal of the disinfectant. These were then placed on a sterile Petri-plate and the explants were trimmed carefully using sterile forceps and scalpel.

The so prepared explants were transferred carefully to nutrient media using sterile forceps. The culture was placed in the incubation chamber for different experiments. Ten replications were done for each treatment in all experiments.

3.1.2.3. Standardization of protocol for direct regeneration of plantlets from aerial stem

Explants (1.0-1.5cm) taken from the middle part (portion containing apical meristem) of the *in planta* aerial stem and aerial stem explants collected from *in vitro* regenerated plants (3-4 month old) were inoculated onto MS medium supplemented with different concentrations and combinations of growth hormones (Table 5). In case of *in vitro* aerial stem explants, the aerial stem was divided into three equal parts i.e., basal, middle and top portion of about 1.0-1.5 cm size.

Observations were recorded from ten random culture tubes, on days taken for regeneration of plantlets, percentage of explants regenerated, number of shoots and roots regenerated from each explant.

3.1.2.3.1. Days taken for regeneration of plantlets

Number of days taken for shoot and root regeneration from each explant was recorded from the date of inoculation to the date of shoot/root regeneration.

3.1.2.3.2. Percentage of explants regenerated

Percentage of explants regenerated was calculated by total number of cultures selected to the number of cultures regenerated.

3.1.2.3.3. Number of shoots regenerated per explant

Total number of shoots regenerated from each explant was counted and the mean calculated.

3.1.2.3.4. Number of roots regenerated per explant

Total number of roots regenerated from each explant was counted and the mean calculated.

3.1.2.4. Standardization of protocol for direct regeneration of plantlets from vegetative buds

Vegetative buds with axillary and apical buds (0.5-1.0 g) were inoculated onto MS medium supplemented with different growth hormones (Table 5).

Observations were recorded from ten random culture tubes, on days taken for regeneration of plantlets, percentage of explants regenerated and number of shoots and roots regenerated from each explant.

3.1.2.4.1. Days taken for regeneration of plantlets

Number of days taken for shoot and root regeneration from each explant was recorded from the date of inoculation to the date of shoot/root regeneration.

3.1.2.4.2. Percentage of explants regenerated

Percentage of explants regenerated was calculated by total number of cultures selected to the number of cultures regenerated.

3.1.2.4.3. Number of shoots regenerated per explant

Total number of shoots regenerated from each explant was counted and mean calculated.

3.1.2.4.4. Number of roots regenerated per explant

Total number of roots regenerated from each explant was counted and mean calculated.

Table 5. Media composition and explants used for *in vitro* direct regeneration of ginger.

Sl. No.	Explant	Growth hormone	Concentration (mgl^{-1})
1	*In planta* aerial stem	TDZ	1.0
		TDZ : IBA	0.5 : 0.5
		TDZ : IBA	1.0 : 0.5
		TDZ : IBA	1.0 :1.0
		TDZ : BAP	0.5 : 0.5
		TDZ : IBA : BAP	1.0 : 0.5 :1.0
		TDZ : IBA : GA_3	0.5 : 0.5 : 0.5
		TDZ : BAP: GA_3	0.5 : 0.5 : 0.5
2	*In vitro* aerial stem	BAP	1.0
		BAP	2.0
		BAP : NAA	1.0 : 0.5
		BAP : NAA	1.0 : 1.0
		BAP : NAA	2.0 : 1.0
		BAP : NAA	3.0 : 1.0
		BAP : NAA	5.0 : 1.0
3	Vegetative bud	BAP : NAA	0.5 : 0.5
		BAP : NAA	1.0 : 0.5
		BAP : NAA	1.0 : 1.0
		BAP : NAA	2.0 : 1.0
		BAP : NAA	5.0 : 0.5

3.1.2.5. Standardization of protocol for callus induction

For callus induction, aerial stem explants (1.0-1.5cm) (*in vitro* and *in planta*) were inoculated onto MS medium supplemented with 2, 4-D at different concentrations (Table 6).

Observations were recorded from ten random culture tubes, on days taken for callus induction, percentage of explants callused, nature of callus – callus colour and texture.

3.1.2.5.1. Days taken for callus induction

Number of days taken for callus induction from each explant was recorded from the date of inoculation to the date of callus induction.

3.1.2.5.2. Percentage of explants callused

Percentage of explants callused was calculated by total number of cultures selected to the number of cultures induced callus.

3.1.2.5.3. Nature of callus

Nature of callus was recorded by noticing colour and texture of the callus.

3.1.2.6. Standardization of protocol for callus regeneration

For callus regeneration, the aerial stem derived calli (2.0-2.5 g) were transferred onto MS medium supplemented with BAP and NAA at different concentrations (Table 6).

Observations were recorded from ten random culture tubes, on days taken for regeneration of plantlets, percentage of cultures regenerated and number of shoots and roots regenerated from each callus culture.

3.1.2.6.1. Days taken for regeneration of plantlets

Number of days taken for shoot and root regeneration from each culture was recorded from the date of inoculation to the date of shoot/root regeneration.

3.1.2.6.2. Percentage of cultures regenerated

Percentage of cultures regenerated was calculated by total number of cultures selected to the number of cultures regenerated.

3.1.2.6.3. Number of shoots regenerated per culture

Total number of shoots regenerated was counted per callus at the size of 1.5 cm^2 from each callus cultures and mean calculated.

3.1.2.6.4. Number of roots regenerated per culture

Total number of roots regenerated was counted per callus at the size of 1.5 cm^2 from each callus cultures and mean calculated.

Table 6. Media composition and explants used for callus induction and regeneration of ginger.

Sl. No.	Experiment	Explant	Growth hormone	Concentration (mgl^{-1})
1	Callus induction	*In planta* and *in vitro* aerial stem	2,4-D 2,4-D 2,4-D	0.5 1.0 2.0
2	Callus regeneration	Calli derived from aerial stem explants	BAP : NAA BAP : NAA BAP : NAA BAP : NAA BAP : NAA	1.0 : 0.5 1.0 : 1.0 2.0 : 1.0 2.0 : 0.5 5.0 : 0.5

3.1.2.7. Standardization of protocol for somatic embryogenesis

Callus cultures derived from aerial stem explants were subcultured onto MS medium containing 2,4-D + BAP in different combinations (Table 7). These cultures were subjected to stress by keeping them without sub culturing for 40 – 60 days, to induce embryogenic callus. The embryogenic calli were then transferred to MS medium containing BAP in different concentrations for induction of somatic embryos.

Observations were recorded from ten random culture tubes, on days taken for somatic embryo induction, percentage of culture responded and type of callus response.

3.1.2.7.1. Days taken for somatic embryo induction

Number of days taken for somatic embryo induction from each callus culture was recorded from the date of inoculation to the date of somatic embryo induction.

3.1.2.7.2. Percentage of culture responded

Percentage of culture responded was calculated by total number of cultures selected to the number of culture responded.

3.1.2.7.3. Type of callus response

Callus response was recorded by noticing the type of the response showed by the callus (somatic embryo induction/ callus multiplication).

3.1.2.8. Standardization of protocol for direct somatic embryogenesis

Aerial stem and leaf explants were inoculated onto MS medium supplemented with TDZ and IBA at different concentrations (0.5: 0.5; 1.0: 0.5; 1.0:1.0 mg l^{-1}) and TDZ alone (0.2; 0.5; 1.0 mg l^{-1}).

Observations were recorded from ten random culture tubes, on days taken for somatic embryo induction, percentage of culture responded and type of callus response.

3.1.2.8.1. Days taken for somatic embryo induction

Number of days taken for somatic embryo induction from each explant culture was recorded from the date of inoculation to the date of somatic embryo induction.

3.1.2.8.2. Percentage of culture responded

Percentage of culture responded was calculated by total number of cultures selected to the number of culture responded.

3.1.2.9. Standardization of protocol for germination of somatic embryos

Mature somatic embryos were transferred to MS medium with BAP + NAA in different combinations (Table 7).

Observations were recorded from ten random culture tubes, on days taken for regeneration of plantlets, percentage of cultures regenerated, number of shoots and roots regenerated from each somatic embryo culture.

3.1.2.9.1. Days taken for regeneration of plantlets

Number of days taken for shoot and root regeneration from each somatic embryo was recorded from the date of inoculation to the date of shoot/root regeneration.

3.1.2.9.2. Percentage of embryo cultures regenerated

Percentage of embryo cultures regenerated was calculated by total number of embryos selected to the number of cultures regenerated.

3.1.2.9.3. Number of shoots regenerated per embryo culture

Total number of shoots regenerated from the selected somatic embryo cultures were counted and mean calculated.

3.1.2.9.4. Number of roots regenerated per embryo culture

Total number of roots regenerated from the selected somatic embryo cultures were counted and mean calculated.

Table 7. Media composition and explants used for somatic embryogenesis and plantlet regeneration in ginger.

Sl. No.	Experiment	Explant	Growth hormone	Concentration (mgl^{-1})
1	Somatic embryogenesis	Callus cultures derived from aerial stem explants	2,4-D : BAP 2,4-D : BAP 2,4-D :BAP 2,4-D : BAP 2,4-D : BAP BAP BAP BAP BAP BAP	0.2 : 0.2 0.5 : 0.2 1.0 : 0.5 1.0 : 1.0 2.0 : 0.5 0.2 0.5 1.0 2.0 5.0
2	Plantlet regeneration from somatic embryos	Matured somatic embryos	BAP : NAA BAP : NAA BAP : NAA BAP : NAA BAP : NAA	0.2 : 0.2 1.0 : 0.5 1.0 : 1.0 2.0 : 0.2 2.0 : 1.0

3.1.2.10. Shoot proliferation

All cultures were transferred to MS medium supplemented with BAP and NAA (1.0:1.0 mgl^{-1}) for shoot and root proliferation. Borosil culture tubes (15 x 200 mm) were used to proliferate callus/somatic embryo regenerated plants whereas Borosil conical flasks (250 ml) were used for proliferating direct regenerated plants (*in vitro* aerial stem and vegetative buds). In the case of the *in planta* aerial stem cultures, the explants having first shoot bud initials were transferred to liquid medium supplemented with different concentrations of TDZ + IBA (02:0.2,1.0:0.5 and 1.0:1.0 mgl^{-1}) and BAP + NAA (0.2: 0.2, 1.0:0.5 and 1.0:1.0 mgl^{-1}) and incubated on a shaker (Cetronat) to hasten the shoot bud proliferation. Then these cultures were again subcultured onto MS solid medium supplemented with BAP and NAA (1.0:1.0 mgl^{-1}).

3.2. Histological studies

Standard procedures (Johansen, 1940) were followed to carry out histological studies.

3.2.1. Fixation of plant tissue

Specimens at different developmental stages were initially washed with distilled water and fixed in FAA (5 ml formalin: 5 ml glacial acetic acid: 90 ml 70% alcohol) for 24 hour.

3.2.2. Dehydration

Before further treatments, the specimens were washed under tap water for 5 hours followed by passing through 5%, 10%, 15%, 20%, 30%, 50% and 70% ethyl alcohol (30 minutes each) and subsequently dehydrated in ethyl alcohol and tertiary butyl alcohol (TBA) series.

40% ethyl alcohol + 10% TBA + 50 ml distilled water (1 hour)

↓

50% ethyl alcohol + 20% TBA + 30 ml distilled water (1 hour)

↓

50% ethyl alcohol + 35% TBA + 15 ml distilled water (1 hour)

↓

50% ethyl alcohol + 50% TBA (1 hour)

↓

75% TBA + 25 ml absolute alcohol (1 hour)

↓

Pure TBA (over night – Three times)

3.2.3. Infiltration and embedding

The specimens were processed for gradual infiltration after complete dehydration. The samples were dipped in a mixture of TBA and melted paraffin wax (56 – 58°C, Qualigens, Mumbai) and kept in oven at 60°C. The proportion of paraffin gradually increased, keeping 2 -3 hours in each and finally the samples were placed in melted pure paraffin overnight. The specimens were transferred to the lower shelf of the oven after giving the final change of pure paraffin. Blocks (with specimens

properly arranged in melted wax) were then prepared and stored in a cool place for further use.

3.2.4. Microtome sectioning and permanent slide preparation

Sections, 10 μm thickness, were cut using 'Ernst Leitz Wetzlar GMBH' (Germany) microtome. They were mounted on the slide and warmed the slide gently to spread the sections uniformly on the slide. The slides were kept for drying in a slanting position.

3.2.5. Dewaxing and staining

To remove the wax adhering to the specimens, the slides were dewaxed with a xylene – alcohol series.

Xylene (10 minutes)

↓

Xylene (7 – 10 minutes)

↓

Xylene + alcohol (3 minutes)

↓

100 % alcohol / TBA (2 minutes)

↓

95% alcohol (2 minutes)

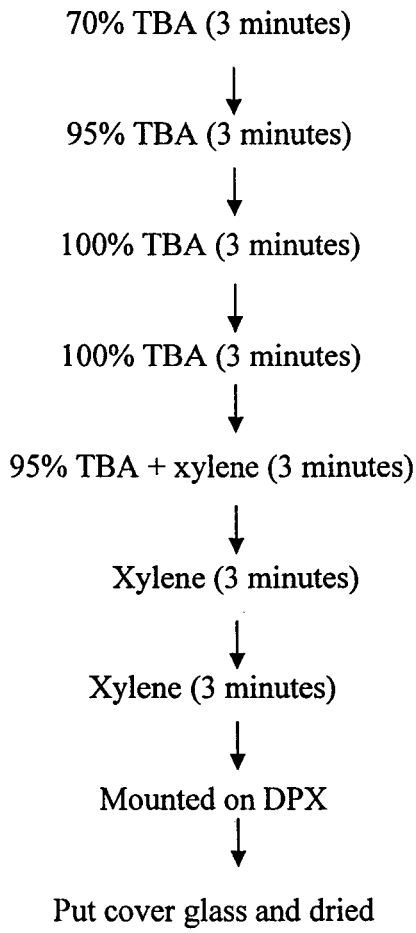
↓

70% alcohol (2 minutes)

↓

Water (dip the slide gently)

De paraffinized sections / slides were then transferred to the Papanicolous staining solution (Harries hematoxyline solution, Qualigens) for 20 minutes and washed in water. The slides were then dehydrated through TBA – xylene series.



3.3. Photography

Photographs of *in vitro* cultures were taken with a 'Nikon D-70'camera. Enlarged photographs of somatic embryos and callus were taken under 'Leica M 27_5' stereo microscope. In histological studies, the slides were examined under an Olympus BX 50 microscope and photographs were taken.

3.4. Genetic fidelity analysis

In order to check the genetic fidelity of the *in vitro* derived plants, molecular characterization was done using RAPD markers. Genomic DNA was extracted from young aerial stem with leaves (2g).

3.4.1. DNA Extraction

3.4.1.1. Preparation of stock solutions for reagents and buffers for DNA extraction

The reagents and buffers for DNA extraction were prepared as per Sambrook *et al.* (1989). The composition of various stock solutions and buffers are given in the Tables 8 and 9.

Table 8. Preparation of stock solutions for DNA extraction.

Sl. No	Solution	Method of preparation
1.	Tris (pH 8.0), 500ml	Dissolve 60.55 g Tris base (Sigma) in 300 ml distilled water. Adjust pH to 8 by adding concentrated HCl. Adjust volume to 500 ml. Dispense to reagent bottles and sterilize by autoclaving.
2	0.5M EDTA (pH 8.0), 500 ml.	Dissolve 93.05 g of EDTA-disodium salt (Sigma) in 300 ml of water. Adjust pH to 8 by adding NaOH pellets. Adjust volume to 500 ml. Dispense into reagent bottles and autoclave.
3.	5M NaCl, 500 ml	Weigh 146.1 g NaCl (Merck) add 200 ml of water and mix well. When the salts get completely dissolved, adjust the final volume to 500ml. Dispense into reagent bottles and autoclave.
4.	3M Sodium acetate (pH5.2), 250 ml	Dissolve 61.523 g of anhydrous sodium acetate (Qualigens) in 200 ml of water and mix well. When dissolved completely adjust the pH of the solution to 5.2 with glacial acetic acid (99- 100%). Dispense to reagent bottles and autoclave.
5.	Ethidium Bromide $10mgml^{-1}$, 100ml	Add 1 g Ethidium Bromide to 100 ml of distilled water. Keep on magnetic stirrer to ensure that the dye has dissolved completely. Dispense to amber coloured reagent bottle and store at 4°C.
6.	70% ethanol, 500 ml	Take 360 ml of ethanol, mix with 140 ml of distilled water. Dispense to reagent bottle and store at 4°C.
7.	Chloroform: isoamyl alcohol (24:1), 500 ml	Measure 480 ml of chloroform and 20 ml of isoamyl alcohol. Mix well and store in reagent bottle in room temperature.

Table 9. Preparation of buffers for DNA extraction.

Sl. No.	Buffer	Method of preparation
1	CTAB Extraction Buffer (1 liter) 100mM Tris HCl (pH 8.0) 20mM EDTA (pH 8.0) 1.4M NaCl 2 % CTAB (w/v) 0.2 % β-mercaptoethanol. (v/v)	Measure 100 ml Tris (1M), 280 ml of NaCl, 40 ml of EDTA (0.5M). Mix with about 400ml of hot distilled water, add 20 g of CTAB to this. Adjust final volume to 1 liter. Dispense to reagent bottles and autoclave. Just before use, add 0.2% β-mercaptoethanol.
2	TE (0.1mM) buffer 100ml 100mM Tris HCl (pH 8.0) 0.1mM EDTA (pH 8.0)	Take 1ml of Tris HCl (1M), 20ml of EDTA (0.5M). Mix with 99ml of sterile distilled water taken in a reagent bottle, mix thoroughly, autoclave.
3	TAE buffer 10x (1 liter)	Weigh 48.4g of Tris base, add 20 ml of EDTA (0.5M), 11.42 ml of Glacial acetic acid and around 150 ml distilled water. Dissolve the salt and adjust volume to 1 liter. Autoclave.
4	Gel loading buffer (6x) 100ml 0.25% Bromophenol blue 30% Glycerol	Dissolve 0.25 g of BPB in 99 ml of 30% Glycerol. Keep on magnetic stirrer for several hours to get the dye completely dissolved. Dispense to reagent bottles and keep in 4°C.

3.4.1.2. Isolation of genomic DNA

CTAB method (Doyle and Doyle, 1987) was followed to isolate DNA from the tissue. The step wise protocol used for extraction of DNA is given below:

1) Grind 2 g of young aerial stem with leaves in liquid nitrogen with a sterilized mortar and pestle and add 8 ml of preheated (60°C) CTAB buffer. Add 0.2 % β – mercaptoethanol.
2) Incubate at 60°C for 1 hour.
3) Extract with equal volume of chloroform: isoamyl alcohol (24:1), mix well for 15 minutes and centrifuge at 10000 rpm for 15 minutes at room temperature.
4) Take the aqueous phase and add 2/3rd volume of ice cold isopropanol.
5) Incubate at 0°C for 1 hour and centrifuge at 10000 rpm for 15 minutes at 4°C.

6) Discard the supernatant and invert the tube on tissue paper for a few minutes then wash the DNA with 80% ethanol and centrifuge at 10000 rpm for 5 minutes.
7) Dry the pellet in vacuum for 15 minutes.
8) Dissolve the pellet in nuclease free water.

3.4.1.3. Purification of genomic DNA

The isolated DNA was purified as below:

1) Add 10 µg / ml of RNAase A and incubated at 37°C for 30 minutes.
2) Add equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1), shake well and centrifuge at 12000 rpm for 15 minutes.
3) Take the aqueous phase and add equal volume chloroform: isoamyl alcohol (24:1), shake well and centrifuge at 12000 rpm for 15 minutes.
4) To the aqueous phase add equal volume of chilled 100 % ethanol and incubate at 0°C for 1 hour centrifuge at 12000 rpm for 15 minutes.
5) Air dry the pellet and dissolve in nuclease free water and estimate the yield.

3.4.1.4. Quantification of DNA

In order to perform RAPD reaction or PCR works the DNA has to be quantified. The most commonly used method of agarose gel electrophoresis was used to quantify the DNA.

3.4.1.4.1. Reagents

The reagents used for electrophoresis are given below:

i) 50 X TAE / 1000 ml (pH – 8)
- 242 g Tris base
- 57.1 ml glacial acetic acid
- 100 ml 0.5 M EDTA (pH – 8)

ii) 10 X loading dye

- Bromophenol blue - 0.25 %
- Xylene cyanol - 0.25 %
- Glycerol - 50 %
- TAE - 1 X
-

iii) Ethidium bromide

- 10 mg ml^{-1} ethidium bromide in double distilled water.

3.4.1.4.2. Protocol

i) Preparation of agarose gel

- Fix the tapes to ends of gel tray. Keep the comps and place the gel tray horizontally.
- Prepare 0.8 % agarose in 1 X TAE (0.8 g agarose in 100 ml 1 X TAE). Boil the solution in microwave oven until all the agarose particles are completely dissolved. Allow to cool (60°C).
- Add 2 µl of ethidium bromide and pour to gel tray.
- After 20 minutes remove the tape and comb, place the gel into the tank and pour 1 X TAE until the gel is fully immersed.
- Load the DNA samples with loading dye (6 X) into the wells with a standard marker DNA.
- Run gel at 50 – 60 volts.
- Visualize the DNA bands on a UV transilluminator and document gel using a Gel Documentation system (Alpha Imager 2200).
- Compare the intensity of the DNA bands of the samples with the intensity of the standard DNA bands (As the amount of DNA present in each standard DNA bands is known, the amount of DNA of each sample can be calculated by comparing the fluorescent yield of the sample with that of λ DNA standard).

3.4.2. RAPD analysis

RAPD reactions were performed as per the method suggested by Williams *et al.* (1990) with minor modifications. The experiments repeat atleast two times.

3.4.2.1. PCR components

- Template DNA concentration – a concentration of 40 ng DNA per 25 µl reaction mix was used.
- Taq DNA polymerase – the enzyme concentration taken is 1 U that is 0.33 µl in 25 µl reaction mix (Bangalore Genei, India).
- dNTPs – final concentrations of 0.2 mM were used (Bangalore Genei, India).
- $MgCl_2$ – a final concentration of 2 mM per 25 µl reaction mix (Bangalore Genei, India).
- Primers – primers of series OPB, OPD and OPJ were used to a final concentration of 10 picomoles in 25 µl reaction mix (Operon Technologies, USA).
- Assay buffer – for PCR reactions, assay buffer with a final concentration of 1 X was used (Bangalore Genei, India).

3.4.2.2. PCR protocol

To a 0.2 ml thin walled PCR tube add the following in order.

- Nuclease free water - 17.42 µl
- 10 X reaction buffer - 2.5 µl
- $MgCl_2$ (50 mM) - 0.25 µl
- dNTP mix (2.5 mM) - 0.5 µl
- Primer (5 pico moles) - 2 µl
- Template DNA - 2 µl
- Taq DNA polymerase (3 U / µl) - 0.33 µl

Total reaction volume is 25 µl. Mix well the mixture and spin down for 10 seconds.

3.4.2.3. Master mix preparation

Since the pipetting of small volumes is difficult and often inaccurate, a master mix was prepared where constituents common to all the reactions are combined in one tube multiplying the volume for one reaction with the total number of samples. Latter, the appropriate amount of the master mix was aliquot to each tube and DNA template (or the variable constituent) was added separately in each tube.

3.4.2.4. Amplification condition

- Denaturation at 94°C for 3 minutes.
- Denaturation at 94°C for 1 minute.
- Annealing at 37°C for 1 minute.
- Extension at 72°C for 1 minute.

  (Step 2 – 4 was repeated 34 times).
- Final extension at 72°C for 10 minutes.
- Refrigerate at 4°C.

3.4.2.5. Primer screening

Primers of OPB, OPD and OPJ series were used for primer screening. Out of 25 RAPD primers screened, 9 primers of OPB series, 4 primers of OPD series and 5 primers of OPJ series were used for RAPD studies. The sequences of the primers are given in the Table. 10.

Table 10. List of RAPD primer sequences used in the study.

Sl. No.	Primer	Sequence (5' – 3')	Sl. No	Primer	Sequence (5' – 3')
1	OPB 01	GTTTCGCTCC	10	OPD 02	GGACCCAACC
2	OPB 06	TGCTCTGCCC	11	OPD 03	GTCGCCGTCA
3	OPB 07	GGTGACGCAG	12	OPD 11	AGCGCCATTG
4	OPB 09	TGGGGGACTC	13	OPD 13	GGGGTGACGA
5	OPB 10	CTGCTGGGAC	14	OPJ 01	CCCGGCATAA
6	OPB 11	GTAGACCCGT	15	OPJ 06	TCGTTCCGCA
7	OPB 14	TCCGCTCTGG	16	OPJ 07	CCTCTCGACA
8	OPB 15	GGAGGGTGTT	17	OPJ 10	AAGCCCGAGG
9	OPB 17	AGGGAACGAG	18	OPJ 16	CTGCTTAGGG

3.4.2.6. Electrophoresis

The amplified products of RAPD were analyzed using 2% agarose gel. The protocol used is given below:

- Prepare 2 % agarose in 1 X TAE (2 g agarose in 100 ml 1 X TAE and 2 µl ethidium bromide) and cast the gel.
- Load the PCR products with 6 X loading dye into the wells. In the first well a standard DNA was also loaded as a marker and run at 60 volts.
- Visualize the DNA bands on a UV transilluminator and document the gel using a gel documentation system (Alpha Imager 2200, Bio Rad, USA).
- Amplified products were scored on the basis of major bands present. Same size bands across the samples were treated as monomorphic bands.

3.5. Hardening

3.5.1. Standardization of protocol for hardening of plantlets

Well rooted plantlets of 3-4 months old were removed from the culture flasks, washed in tap water to remove the adhering medium and transplanted onto a hardening medium disposable polythene cups (250 ml), irrigated and kept covered

with polythene bags (25x15 cm) to maintain humidity, if necessary. Hardening of the tissue cultured plants were done at room temperature. After 2 weeks of hardening the plantlets were transferred to field condition or experimental shed.

To standardize the best hardening medium, different potting mixtures were tried as below:

1) Sterilized coir dust
2) Sterilized coir dust + covering of plantlets with polythene cover (25 x 15cm)
3) Sterilized coir dust + *Trichoderma* @ 5 g/cup (CFU = 10^{14} g^{-1})
4) Soil: sand: coir dust: cow dung (sterilized)
5) Soil: sand: coir dust: cow dung (sterilized): *Trichoderma* @ 5 g/cup

Each treatment was replicated 10 times.

Soil, sand, coir dust and cow dung were sterilized before use, by autoclaving at 120°C for 1 hr at 1.06 Kg cm^{-2}.

3.5.2. Lay out

The experiment was laid out in a randomized block design with five replications. Observation on survival of plants, plant height, number of leaves and chlorophyll content in leaf tissues were taken from 5 randomly selected plants after one month.

3.5.2.1. Plant survival

Plant survival was recorded by counting the number of plants survived out of total number of plants planted.

3.5.2.2. Plant height

Plant height was measured from the base to the tip of the main aerial stem in cm and the mean calculated.

3.5.2.3. Number of leaves

Total number of leaves present in the main tiller was counted and the mean calculated.

3.5.2.4. Chlorophyll estimation

Chlorophyll estimation study was done (Arnon, 1949 and Witham *et al.*, 1971) as below:

Leaf samples were collected from the hardened plants after 30 days of planting (4^{th} or 5^{th} leaf from the top of each plant).

- Grind 0.1 g of leaf tissue in 2 ml of 70% acetone using a mortar and pestle.
- Make up to 5 ml with 70 % acetone
- Centrifuge and take the supernatant (5000 rpm for 5 minutes)
- Make up to 10 ml with 70 % acetone
- Centrifuge and take the supernatant (5000 rpm for 5 minutes)
- Make up to 15 ml with 70 % acetone
- Take spectrophotometric reading at 663 and 645 nm.

3.5.2.4.1. Calculation

The amount of chlorophyll present in the plant tissue extract (mg chlorophyll g $tissue^{-1}$) was calculated using the following formula:

$$\text{Mg chlorophyll}^{a}\ \text{g tissue}^{-1} = 12.7\ (A_{663}) - 2.69\ (A_{645}) \times \frac{V}{1000 \times W}$$

$$\text{Mg chlorophyll}^{b}\ \text{g tissue}^{-1} = 22.9\ (A_{645}) - 4.68\ (A_{663}) \times \frac{V}{1000 \times W}$$

$$\text{Mg total chlorophyll g tissue}^{-1} = 20.2\,(A_{645}) + 8.02\,(A_{663}) \times \frac{V}{1000 \times W}$$

3.6. Field evaluation

3.6.1. Planting of tissue cultured plants

Planting of the tissue cultured plants were done under protected condition, in an experimental shed of 24 m X 6 m size with 50% shade. The hardened plantlets were transferred to polythene bags (30 x 40 cm) containing a mixture (approximately 4 Kg) of garden soil, sand, coir dust and cow dung (2:1:1:1) + 10g Trichoderma (CFU = 10^{14} g^{1}). Minimum 20 plants were planted in each group of variety Jamaica and variety Varada (Table 11). The crop duration was July to February in all the three years.

Table 11. Field evaluation of *in vitro* regenerated plants - treatments and growing conditions.

Sl. No.	Planting material	Growing conditions
1	Plantlets regenerated from aerial stem	Single plantlet was planted in each polythene bag (30 x 40 cm) containing a mixture of garden soil, sand, coir dust and cow dung (2:1:1:1) + 10g Trichoderma (CFU = 10^{14} g^{-1}).
2	Plantlets micro propagated from vegetative bud	
3	Plantlets regenerated from callus	
4	Conventionally propagated plants	

Mulching was done immediately after planting with green leaves. Approximately @ 200g bag^{-1}. The plants were raised under organic practice applying dried and powdered cowdung @ 200 g twice after planting and with life saving irrigation.

3.6.2. Lay out

The experiment was laid out in a Completely Randomized Design (CRD). Observations on plant height, number of tillers, number of leaves, leaf length, leaf width, number of cormlets per rhizome, length and circumference of cormlets,

internodes per cormlet, internodal length and weight of rhizome from ten randomly selected plants of each group. All aerial morphological characters recorded regularly at one month interval. The rhizome characters were recorded after harvest. The data taken at full maturity was subjected to Analysis of Variance.

3.6.2.1. Plant height

Height of the main tiller was recorded in centimeters. The height of the aerial stem from the soil surface to the tip was considered.

3.6.2.2. Number of tillers

Total number of tillers per plant was recorded.

3.6.2.3. Number of leaves

Number of leaves present in the main tiller was recorded.

3.6.2.4. Leaf length

Length of 3rd and 4th leaves from the top was recorded in centimeters from tip to leaf base and the mean value calculated.

3.6.2.5. Leaf width

Width of 3rd and 4th leaves was recorded in centimeters, from the maximum width region and the mean value calculated.

3.6.2.6. Number of cormlets

Total number of distinguishable rhizome branches present in a whole clump was recorded.

3.6.2.7. Length and circumference of cormlet

Length and circumference of 4 cormlets in each rhizome were recorded in centimeters and the mean value was computed.

3.6.2.8. Internodes per cormlet

Total number of internodes present in 4 cormlets in each rhizome were recorded and the mean value was calculated.

3.6.2.9. Internodal length.

Length of internodes present in 4 cormlets was recorded in centimeters and the mean value was considered.

3.6.2.10. Weight of rhizome

The whole weight of fresh rhizome was recorded in grams after removing the adhering roots.

3.6.3. Evaluation of second generation plants

For the evaluation of tissue cultured ginger plants in the second generation, rhizomes obtained from the first generation plants were planted in the experimental shed as described in Section (3.6.1). Ten plants per treatment were kept. The experimental lay out and observations were same as described in Section (3.6.2.).

3.6.4. Yield evaluation of tissue cultured plants in large bed

The tissue cultured ginger plants of var. Varada was also evaluated in large single bed (3 m^2) at IISR experimental farm, Peruvannamuzhi, under field conditions. A total of 60 plants were planted in one bed at a spacing of 20x25 cm (non replicates). The crop was raised under organic practice using farm yard manure @ 15 Kg^{-1} bed. The planting was done in the month of September 2005. Observations were recorded on plant height, number of tillers, number of leaves, number of leaves, leaf length, leaf width, weight of rhizome, number of cormlets per rhizome, length and circumference of cormlets, internodes per cormlet and internodal length from ten randomly selected plants as detailed under Section 3.6.2.

3.7. Quality evaluation

The harvested rhizomes of each group (plants regenerated from aerial stem, plants regenerated from vegetative bud, plants regenerated from callus and conventionally propagated plants-first generation) were pooled separately and dried. From the dried rhizomes five samples were selected for quality analysis.

3.7.1. Dry recovery

Peeled rhizomes were weighed and record the fresh weight. Then the rhizomes were dried under sunlight for 2 weeks and record the dry weight and the percentage recovery was computed as below:

$$\text{Dry recovery (\%)} = \frac{\text{Dry weight}}{\text{Fresh weight}} \times 100$$

3.7.2. Volatile oil

To determine the water insoluble steam volatile oil, modified Clevenger trap method was used (AOAC, 1975).

3.7.2.1. Apparatus

- Flasks, short neck, round bottom type, 1 or 2 liter, with S.T. 24/40 ground joint.
- Heat source: Electric heating mantle
- Variable voltage transformer (if electric heating mantle is used).
- Volatile oil traps, Clevenger type with S.T. 24/40 ground joints – Lighter than water design for oils with densities less than that of water.
- West type container, 400 mm in length with water cooled drip tip and
  S.T. 24/40 ground joints.

3.7.2.2. Preparation of samples

25 g of dried ginger rhizome was ground to fine powder using a hand operated mill.

3.7. 2.3. Procedure

- Weigh accurately 20 g of sample and transferred to the flask, using 500 ml of water to aid quantitative transfer.
- Add 500 ml of water to the flask.
- Assemble the apparatus correctly using the proper Clevenger trap.
- Heat the flask in electric heating mantle and maintain a distillation rate of 1 – 1.5 drop of oil per second.
- Distill until two consecutive readings taken at one hour intervals show no change of oil volume.
- Cool to room temperature – allow to stand until the oil layer is clear and read the volume of the oil collected, estimated to the nearest 0.02 ml.

3.7. 2.4. Calculation

$$\text{Volatile oil \% (v/w)} = \frac{\text{Vol. of oil}}{\text{Wt. of samples (g)}} \times 100$$

3.7.3. Oleoresin

To estimate the oleoresin quantity, acetone extraction was used (ASTA, 1968).

3.7.3.1. Apparatus

Chromatographic column with stop cock (18 x 450 mm).

3.7.3.2. Sample material

A mixture of 50 ml acetone and 10 g of ginger powder.

3.7.3.3. Procedure

Weigh 10 g of sample and transferred to the Chromatographic column. Add 50 ml of acetone. Allow to stand overnight or 16 hrs at 25±2°C. Filter extract through non absorbent cotton. Collect the filtrate to a 100 ml beaker (before collecting the filtrate initial weight of the beaker should be recorded) and allowed to dry in a hot air oven. Difference in the initial and the final weights of the beaker gives the amount of oleoresin.

3.7.3.4. Calculation

$$\text{Acetone extract \% (v/w) (oleoresin)} = \frac{\text{Wt. of residues}}{\text{Wt. of samples (g)}} \times 100$$

3.7.4. Crude fiber

To determine the organic matter in the dried residues remaining after digesting the sample with boiled H_2SO_4 and NaOH (ASTA, 1968).

3.7.4.1. Apparatus

The fiber extraction was carried out in a 'Dosi Fibre' unit of J.P. Selecta Spain.

3.7.4.2. Sample material

2 g of (grounded) ginger powder.

3.7.4.3. Reagents

H_2SO_4	.25 g in 2 liter
NaOH	- 25 g in 2 liter
Ethyl alcohol	- 80%.

3.7.4.4. Procedure

Weigh 2 g of coarsely ground dry ginger sample into the crucible (initial weight of the crucible should be recorded). Attach it to the extraction unit. Digest the sample with 200 ml of the sulphuric acid solution at 100°C. Boil in acid for 30 minutes. After 30 minutes wash with boiling water until washings were neutral. Then add 200 ml of sodium hydroxide solution and again connect the flask to the condenser and boil for 30 minutes. After 30 minutes wash with boiling water till the washings were neutral. Wash with 80% ethyl alcohol and allowed to dry in a hot air oven. Difference in the initial and the final weights of the crucible gives the weight of crude fibre.

3.7.4.5. Calculation

$$\text{Crude fibre \% (v/w)} = \frac{\text{Wt. of residues}}{\text{Wt. of samples}} \times 100$$

3.7.5. Estimation of Starch

Starch was estimated by Anthrone Reagent method described by Hedge and Hofreiter (1962).

3.7.5.1. Reagents

1) Anthrone Reagent
2) 80% ethanol
3) 52% perchloric acid
4) Standard dextrose stock solution – 100 mg in 100 ml with distilled water
5) Working standard – 10 ml of stock was diluted to 100 ml with distilled water.

3.7.5.2. Procedure

100 mg of sample was weighed and then treated with 80% ethanol to remove sugars. Centrifuged and retained the residue. The residue was dried over a water bath.

Then add 5 ml of 52 % perchloric acid and centrifuged at 5000 rpm for 20 minutes and save the supernatant. Repeat the extraction using fresh perchloric acid and again centrifuge. Finally the supernatant was made up to 100 ml.

3.7.5.3. Estimation

Pipette out 0.2 ml of the sample into a series of the test tube and make up the volume 1 ml with distilled water. Add 4 ml of Anthrone Reagent to each tube. Appropriate standard and blank were also done similarly. The absorbance was measured at 630 nm.

3.7.6. Estimation of total carbohydrate

Total carbohydrate was estimated by Phenol Sulphuric Acid method described by (Dubois *et al.*, 1956; Krishnaveni *et al.*, 1984).

3.7.6.1. Reagents

1) 5% Phenol
2) 96% Sulphuric acid
3) Standard glucose stock – 100 mg of glucose in 100 ml of distilled water
4) Working standard -10 ml of stock was diluted to 100ml with distilled water.

3.7.6.2. Procedure

100 mg of the sample was taken in a boiling tube. It was hydrolyzed by keeping in a boiling water bath for 3 hours with 5 ml of 2.5 N HCl and cooled to room temperature. Neutralize it with solid sodium carbonate until the effervescence ceases. Make up the volume to 100 ml and centrifuge.

3.7.6.3. Estimation

Pipette out 0.2 ml of the sample solution into a series of test tubes. Make up the volume in each test tube to 1 ml with distilled water. Add 1 ml of 5% Phenol solution and 5 ml of 96% sulphuric acid to each test tube and shake well. Appropriate standard and blank were also done similarly. After 10 minutes shake well the contents in tube and keep it in water bath for 20 minutes at 25 – 30°C. The absorbance was measured at 490 nm.

3.8. Statistical analysis

3.8.1. Analysis of variance

3.8.1.1. Completely Randomized Design

The data on *in vitro* studies and field evaluation were analyzed as per complete randomized design (CRD) (Panse and Sukhatme, 1985).

3.8.2. Randomized Block Design

The data on the hardening trial were subjected to analysis of variance as per randomized block design (RBD) (Panse and Sukhatme, 1985).

The quality evaluation data were transformed using Arcsin √Percentage transformation and the data were analyzed as per Randomized Block Design (RBD).

3.8.3. Correlation and Path analysis

Pooled data on yield and yield attributes (Section 3.6) were subjected to correlation and path analysis (Dewey and Lu, 1959; Singh and Chaudhary, 1985).

INVESTIGATION ON DIRECT *IN VITRO* SHOOT REGENERATION FROM AERIAL STEM EXPLANT OF GINGER (*Zingiber officinale* Rosc.) AND ITS FIELD EVALUATION

Thesis submitted to the

University of Calicut

For the award of degree

of

Doctor of Philosophy

(BOTANY)

by

Lincy A K

University of Calicut

Calicut, Kerala, India

2007

Results and Discussion

4. Results and Discussion

4.1. *In vitro* studies

The effect of genotypes and hormones on *in vitro* responses of the explants is discussed below:

4.1.1. Direct regeneration of plantlets from in planta *aerial stem explants*

4.1.1.1. Days taken for regeneration of plantlets

The explants took nearly 15-18 days for exhibiting first signs of shoot bud initiation. Root regeneration was observed after 25- 30 days of culturing. In this case, no significant difference was observed between the two varieties.

4.1.1.2. Number of shoots

Analysis of variance for shoot regeneration revealed significant effect of the varieties, hormones and variety X hormone interaction for shoot development (Table 12).

Table 12. Analysis of variance for shoot and root regeneration from *in planta* aerial stem.

Source	Degrees of freedom	No. of shoots			No. of roots		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Variety	1	49.51	49.51	4.93*	28.90	28.90	5.97*
Hormone	7	2044.24	292.04	29.10**	1149.20	164.17	33.89**
Variety x Hormone	7	245.94	35.14	3.50**	232.90	33.27	6.87**
Error	144	1445.30	10.04		697.40	4.84	
Total	159	3784.99			2108.40		

Percentage of shoot regeneration and mean number of shoots observed in the 2 varieties under the different hormone combinations are presented in Table 13. Shoot regeneration was observed in all the hormone combinations tried but the percentage of shoot inducing cultures ranged from 70-100% in the var. Jamaica and 60 – 100% in the var. Varada (Table 13). Among the different hormone combinations tried, TDZ + IBA (1:0.5 mgl^{-1}) showed maximum shoot regeneration percentage in the var. Jamaica and TDZ + IBA (1:1 mgl^{-1}) in the var. Varada.

Table 13. *In vitro* shoot regeneration from *in planta* aerial stem on MS medium fortified with different growth hormones.

Growth regulators in basal medium (mg l^{-1})	Jamaica			Varada			Mean	
TDZ : IBA : BAP : GA_3	% of shoot inducing culture	No. of shoots	No. of roots	% of shoot inducing culture	No. of shoots	No. of roots	Shoots	Roots
0.5 : 0.5 : 0.0 : 0.0	80	3.8	7.1	90	9.8	5.4	6.8	6.3
1.0 : 0.5 : 0.0 : 0.0	100	7.6	7.5	90	9.9	6.1	8.8	6.8
1.0 : 1.0 : 0.0 : 0.0	90	11.1	11.4	100	14.6	4.8	12.9	8.1
1.0 : 0.5 : 1.0 : 0.0	90	7.9	4.1	90	6.9	6.0	7.4	5.05
0.5 : 0.5 : 0.0 : 0.5	90	6.0	3.2	90	3.9	3.2	5.0	3.2
0.5 : 0.0 : 0.5 : 0.0	80	1.9	1.2	70	2.2	1.5	2.05	1.4
0.5 : 0.0 : 0.5 : 0.5	90	4.1	1.7	80	4.2	2.0	4.15	1.9
1.0 : 0.0 : 0.0 : 0.0	70	1.1	0.0	60	0.9	0.4	1.0	0.2
Mean		5.4	4.5		6.6	3.7		
CD (P=0.05)								
Variety		0.99	0.69					
Hormone		1.99	1.38					
Variety X Hormone		2.81	1.95					

Mean values for varieties indicated that maximum number of shoots were observed in the var. Varada. Palai *et al.* (2000) reported that the rate of shoot bud regeneration varied with different genotypes.

Among the 8 growth hormone combinations tried, TDZ + IBA (1:1 mgl^{-1}) gave maximum number of shoots in both the varieties. In this medium, the var. Jamaica produced 8-19 shoots (with a mean number of 11.1) and the var. Varada

produced 11-22 shoots (mean number 14.6) after 60 days of culture (Fig. 1.a & b). Well developed plantlets could be obtained after subcultured onto the medium containing BAP and NAA (1:1 mgl^{-1}) (Fig. 1.c).

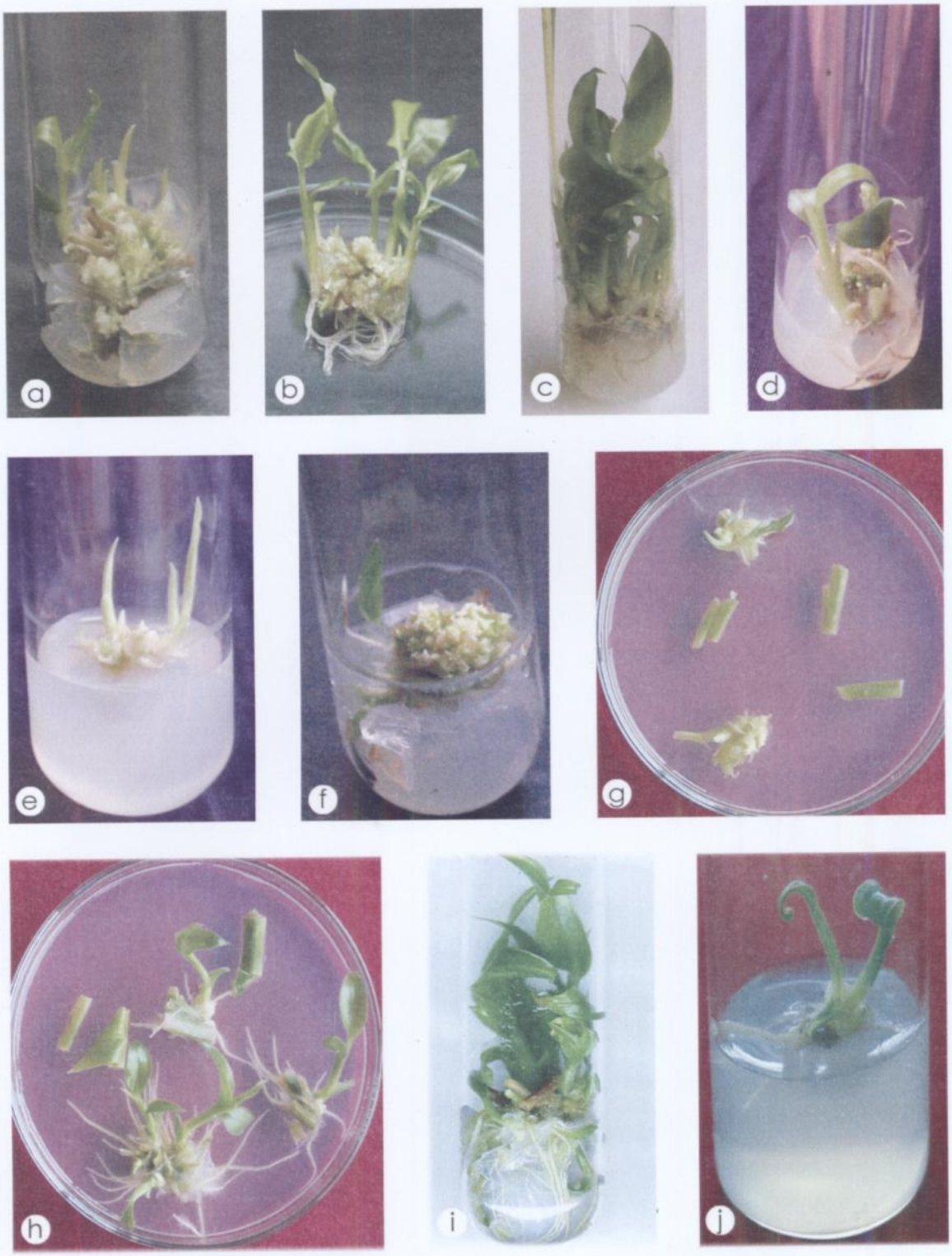


Fig. 1. a & b). Multiple shoot induction from *in planta* explants of var. Jamaica and Varada (MS medium + 1 TDZ + 1 IBA mgl^{-1}). c). Well developed plantlets regenerated from *in planta* aerial stem explant (1 BAP + 1 NAA mgl^{-1}). d). Poor shoot regeneration from *in planta* aerial stem explant cultured on the medium containing TDZ (1 mg l^{-1}). e & f). Shoots regenerated from cultures containing GA3 (0.5 TDZ + 0.5 IBA + 0.5 GA3 mg l^{-1}) -long shoots as compared to other hormone combination (1 TDZ + 1 IBA mgl^{-1}). g). Shoot bud development from *in vitro* aerial stem explant. h). Effect of explant sources - basal, middle and top portions of the *in vitro* aerial stem. i). Shoot proliferation from *in vitro* aerial stem explant of var. Jamaica cultured on BAP + NAA (2:1mgl^{-1}). j). Poor shoot regeneration from *in vitro* aerial stem explant cultured on the medium containing BAP (2 mg l^{-1}) alone.

Cultures containing TDZ and IBA alone or in combination with other hormones showed high multiple shoot induction as compared to other combinations. However, explants inoculated onto medium supplemented with TDZ (1 mgl^{-1}) alone produced lowest number of shoots (Fig. 1.d and Table 13).

TDZ has been shown to exhibit stronger effect than other conventionally used cytokinins in a wide range of species (Mok *et al.*, 1979; Thomas and Katterman, 1986). Effective axillary shoot proliferation and adventitious shoot organogenesis have been reported to be caused by TDZ (Huetteman and Preece, 1993).

Though aerial stem branching is not a general feature in ginger, stressed ginger plants are reported to produce adventitious shoots from the aerial stem possibly due to the change in the endogenous level of hormones caused by the stress (Sasikumar, 2005).

TDZ may be involved in the synthesis or in accumulation of cytokinins in plant tissues (Capelle *et al.*, 1983; Carvalho *et al.*, 2000). This may be the reason for the high multiple shoot induction observed.

Nature of growth regulators affects not only the number of shoots but also the morphology of plantlets. Shoots regenerated form cultures containing GA_3 showed long shoots (visual observations) as compared to other hormone combinations (Fig. 1.e & f). For differentiation and elongation of shoot buds, the role of GA_3 is well acknowledged and it is fundamental for the good continuous growth of *in vitro* regenerated buds (Arous *et al.*, 2001). Mohamed *et al.* (2006) reported that medium supplemented with GA_3 in combination with IBA and $AgNO_3$ was suitable for shoot elongation.

4.1.1.3. Number of roots

Both genotype and hormone influences were significant for root regeneration. Significant interaction was also observed between the varieties and the hormones in this case too (Table 12).

Mean number of roots observed in the two varieties under different hormone combinations are presented in Table 13. The mean values for varieties showed that highest number of roots were observed in the var. Jamaica (Table 13). In this case too, MS medium supplemented with TDZ + IBA (1:1 mgl^{-1}) was found to be the most suitable recipe for root regeneration. The number of roots ranged from 9 to 15 with a mean number of 11.4. In the case of the var. Varada, highest number of roots were observed in MS medium supplemented with TDZ + IBA (1:0.5 mgl^{-1}). The number of roots ranged from 2 to 13 with a mean number of 6.1 (Table 13).

In cultures containing TDZ alone, root regeneration was not observed in the var. Jamaica but in the var. Varada, 20% of cultures showed root regeneration (Table 13).

Bhagyalakshmi and Singh (1988) have reported that IBA was more effective than NAA for root regeneration in meristem culture of ginger.

4.1.2. Direct regeneration of plantlets from in vitro *aerial stem.*

4.1.2.1. Days taken for regeneration of plantlets

In vitro aerial stem explants produced root initials followed by shoot initials from second week of culturing in both the varieties studied. The explants took nearly 7 to 10 days for root initiation and 15 days for shoot bud development in the var. Jamaica. In the var. Varada, the *in vitro* aerial stem took approximately 10 days and 20 days for shoot bud development (Fig. 1.g). Among the different explant sources tried, only in this explants root development prior to shoot development was observed. This may be due to the immature stage of *in vitro* aerial stem explant

compared to the other explants used in the study. George (1993) reported that cuttings taken from the micropropagated plants often produced adventitious roots more readily than normal cuttings.

In addition to this, the wounding caused during explant preparation also reported to enhance root formation prior to shoot formation probably due to the increased endogenous auxin secretion resulted by wounding (George, 1993a). This author also reported that the explants exposed to auxin before transplantation onto rooting media produced roots more readily. Since the *in vitro* aerial stem explants were taken from micropropagated plants, the residual effect of auxin also may cause fast rooting in this case.

4.1.2.2. Effect of explant sources

The mean number of shoots and roots regenerated from different explant sources and effect of various growth hormones are given in the Table 14. Out of the three different explant sources tried (basal, middle and top portions of the aerial stem), basal portions followed by middle portions gave good result (Fig. 1.h). No morphogenesis was observed in the explants taken from the top portions of aerial stem.

The explants containing the axillary buds (basal portion) produced more shoots than the explants containing apical bud (middle portion).

Table 14. Effect of hormones and explant position on shoot and root regeneration in ginger.

Growth regulators in basal medium (mgl^{-1})	Explant (aerial stem cuttings)			
	Basal portion (axillary meristem)		Middle portion (apical meristem)	
BAP : NAA	Shoot initiation	Root initiation	Shoot initiation	Root initiation
1.0 : 0.5	2.0	6.4	0.8	2.6
1.0 : 1.0	3.4	6.8	1.4	3.0
2.0 : 1.0	3.8	6.8	1.6	4.8
3.0 : 1.0	3.2	5.4	1.2	4.2
5.0 : 1.0	3.5	5.6	1.2	3.8
1.0 : 0.0	0.6	2.6	0	0.4
2.0 : 0.0	0	3.2	0.2	0

Among the seven hormone combinations tried, BAP + NAA (2:1 mgl^{-1}) initiated maximum number of shoots and roots in both the basal and middle portions of aerial stem.

According to George (1993), variation in competence can be found between explants from different organs and also between tissues within the same organ. It was found so in the case of ginger, where explants and tissues within the explants differ in their response. The difference may be due to the endogenous content of the plant growth substances, age of the explant tissue and degree of differentiation of tissues.

4.1.2.3. Number of shoots

Genotypic and hormonal effects for the number of shoots were highly significant. The interaction between these factors was also found significant (Table 15).

Table 15. Analysis of variance for shoot and root regeneration from *in vitro* aerial stem.

Source	Degrees of freedom	No. of shoots			No. of roots		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Variety	1	6.43	6.43	8.44**	64.46	64.46	6.05*
Hormone	6	197.07	32.85	43.11**	6800.37	1133.40	106.36**
Variety x Hormone	6	10.47	1.75	2.291*	111.69	18.61	1.75^{ns}
Error	126	96.00	0.76		1342.70	10.66	
Total	139	309.97			8319.22		

Percentage of shoot regeneration and mean number of shoots observed in the 2 varieties under the different hormone combinations are presented in Table 16. Plantlets regeneration was observed in all the combinations of BAP + NAA though the response was varied in the two varieties studied. In the var. Jamaica, hundred percent regeneration was observed in all the combinations of BAP + NAA. But only 80 to 100 % regeneration could be obtained in the var. Varada. Maximum regeneration was observed with BAP + NAA (3:1 mgl^{-1}) in case of the var. Varada. Cultures containing BAP alone showed the least percentage of regeneration in both the varieties (Table 16).

Table 16. *In vitro* shoot and root regeneration from *in vitro* aerial stem on MS medium fortified with different growth hormones.

Growth regulators in basal medium (mgl^{-1})	Jamaica			Varada			Mean	
BAP : NAA	% of shoot inducing culture	No. of shoots	No. of roots	% of Shoot inducing culture	No. of shoots	No. of roots	Shoot	Root
1.0 : 0.5	100	2.7	14.4	90	1.8	10.5	2.3	12.5
1.0 : 1.0	100	2.2	15.2	90	2.2	14.1	2.2	14.7
2.0 : 1.0	100	4.4	18.3	90	2.7	15.1	3.6	16.7
3.0 : 1.0	100	2.7	17.2	100	3.4	14.7	3.1	16.0
5.0 : 1.0	100	2.8	15.5	80	1.6	16.7	2.2	16.1
1.0 : 0.0	30	0.4	0.9	10	0.2	0.0	0.3	0.5
2.0 : 0.0	20	0.7	0.8	40	0.5	0.0	0.6	0.4
Mean		2.3	11.8		1.8	10.2		
CD (P=0.05)								
Variety		0.29	0.60					
Hormone		0.55	2.05					
Variety X Hormone		0.77	2.89					

Among the two varieties studied, the var. Jamaica produced maximum number of shoots as compared to the var. Varada.

Differential response of explants originated from different genotypes of 'Mc Intosh' apple was reported by Looney (1988), suggesting that variation in the endogenous auxin – cytokinin levels in the different genotypes may be responsible for the difference in performance or *in vitro* uptake of exogenous cytokinin (Marino, 1988). The differences in *in vitro* uptake of exogenous cytokinins lead to differences in the endogenous balance of auxin and cytokinin (Alvarez *et al.*, 1989) and hence the difference in response.

In vitro aerial stem produced multiple shoots in all the combinations of BAP and NAA but the number of shoots regenerated varied significantly in the different levels of hormones (Table16). Among seven hormone combinations studied, BAP + NAA (2:1 mgl^{-1}) gave maximum number of shoots in the case of the var. Jamaica. The number of shoots ranged from 3 to 6 with a mean number of 4.4 (Fig. 1.i). In the var. Varada, BAP + NAA (3:1 mgl^{-1}) was found to be most suitable for shoot regeneration. The number of shoots ranged from 2-6 with a mean number of 3.4 after 45 days of culture. High concentrations of BAP and NAA generally resulted in poor shoot/root regeneration.

Aerial stem and decapitated crown sections taken from *in vitro* ginger plants produced multiple shoots when cultured onto MS medium supplemented with BAP + NAA in different concentrations (Ikeda and Tanabe, 1989; Lincy *et al.*, 2004).

Explants inoculated onto the medium containing BAP alone in two concentrations (1 and 2 mgl^{-1}) showed low rate of response (Fig. 1.j). The plantlets obtained from these cultures were also found very weak in both the varieties.

Presence of cytokinin in the medium, preferably in low concentrations, positively affected shoot development in ginger. The endogenous level of cytokinin along with the cytokinin supplemented in low concentrations may reach the optimum level for maximum shoot development. Further increase in cytokinin levels may not result in a corresponding increase in the number of shoots (Samsudeen, 1996).

Cytokinins are generally considered very effective in promoting direct or indirect shoot initiation. They encourage axillary bud growth and reduce apical dominance (George, 1993b). Generally BAP is most effective for meristem, shoot tips and bud cultures (Hu and Wang, 1983). Most of the earlier workers used BAP for shoot multiplication in ginger (Hosoki and Sagawa, 1977; Bhagyalakshmi and Singh, 1988; Sakamura and Suga, 1989; Balachandran *et al.*, 1990; Nirmal Babu *et al.*, 1992a).

The balance between the auxin and cytokinin is important in the initiation and multiplication of shoots. Earlier workers also reported that BAP and NAA combinations were suitable for shoot induction and multiplication in ginger (Sakamura *et al.*, 1986; Charlwood, 1988; Sakamura and Suga, 1989; Samsudeen, 1996).

The positive effect of BAP and NAA on shoot regeneration was reported in other members of Zingiberaceae also (Reghunath and Priyadarshan, 1993, Illg and Faria, 1995; Miachir *et al.*, 2004).

4.1.2.4. Number of roots

The number of roots varied significantly with varieties and levels of hormones. Eventhough, the genotypic and hormone influences were significant for root regeneration, the interaction between genotype and hormone was not significant (Table 15).

Mean number of roots observed in the 2 varieties under the different hormone combinations are given in Table 16. Mean number of roots for the varieties showed that the var. Jamaica produced more number of roots as compared to the var. Varada as observed in case of the shoots. Among the seven hormone combinations studied BAP and NAA (2:1 mgl^{-1}) gave maximum number of roots in the var. Jamaica and BAP and NAA (5:1 mgl^{-1}) in the case of the var. Varada. The number of roots ranged from 15 to 23 with a mean number of 18.3 (var. Jamaica) and 8 to 24 with a mean number of 16.7 (var. Varada) after 45 days of culturing, in these treatments.

Explants cultured in medium containing BAP alone showed poor root regeneration. High concentration of BAP was reported to inhibit root development in ginger (Samsudeen, 1996).

4.1.3. Direct regeneration from vegetative buds

4.1.3.1. Days taken for regeneration of plantlets

The explants took nearly 15 days for shoot bud initiation (Fig. 2.a) and about 20 days for root initiation in the var. Jamaica. In the var. Varada, shoot and root regeneration were observed after 14 and 16 days of culturing, respectively.

Fig. 2. a). Shoot bud regeneration from vegetative bud explant of var. Jamaica (1 BAP + 1 NAA Mg l^{-1}). b). Multiple shoot induction from vegetative bud explant of var. Varada (1 BAP + 1 NAA Mg l^{-1}). c). Callus induced from *in vitro* aerial stem explant - hard, nodular and yellowish in colour with small hairy structures (Type I). d). Callus induced from *in planta* aerial stem - soft, sticky and friable with pale white or creamy colour (Type II). e). Type I callus showing rhizogenesis after 7 days of culturing. f). Type II callus showing green colour. g). Type II callus regeneration (closeup view). h & i). Shoot and root regeneration from callus cultures of var. Jamaica (1 BAP + 1 NAA mgl^{-1}) and var. Varada (2 BAP + 0.5 NAA mgl^{-1}). j). Shoot proliferation in the medium containing 1 BAP + 1 NAA mg l^{-1}.

4.1.3.2. Number of shoots

Analysis of variance for number of shoots showed significant effect of hormones only on shoot development. The effects of varieties and variety X hormone interaction were non significant (Table 17).

Table 17. Analysis of variance for shoot and root regeneration from vegetative bud.

Source	Degrees of freedom	No. of shoots			No. of roots		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Variety	1	5.29	5.29	3.15 ^{ns}	51.84	51.84	3.35 ^{ns}
Hormone	4	98.86	24.72	14.72**	380.70	95.18	6.15**
Variety x Hormone	4	4.86	1.22	0.72 ^{ns}	64.66	16.17	1.05 ^{ns}
Error	90	151.10	1.68		1391.80	15.46	
Total	99	260.11			1889.00		

Percentage of shoot regeneration and mean number of shoots observed in the 2 varieties under the different hormone combinations are presented in Table 18. Shoot regeneration was observed in all the combinations tried but the percentage of shoot inducing cultures varied with different levels of hormones. The percentage ranged from 70 to 100 % in both the verities (Table 18). Maximum regeneration percentage was observed in the combination of BAP + NAA (1:1 mgl^{-1}) in case of the var. Jamaica while BAP + NAA (1:0.5; 1:1 & 2:1 mgl^{-1}) gave 100 percent regeneration in the var. Varada.

Table 18. *In vitro* shoot and root regeneration from vegetative buds on MS medium fortified with different growth hormones.

Growth regulators in basal medium (mgl^{-1})	Jamaica			Varada			Mean	
BAP : NAA	% of shoot inducing culture	No. of shoots	No. of roots	% of shoot inducing culture	No. of shoots	No. of roots	Shoot	Root
0.5 : 0.5	90	2.0	6.4	90	1.8	8.5	1.9	7.5
1.0 : 0.5	90	2.1	10.2	100	3.0	11.0	2.6	10.6
1.0 : 1.0	100	3.8	11.6	100	4.8	15.6	4.3	13.6
2.0 : 1.0	90	3.5	9.6	100	3.8	10.8	3.7	10.2
5.0 : 0.5	70	1.6	11.1	70	1.9	10.2	1.8	10.7
Mean		2.6	9.8		3.1	11.2		
CD (P=0.05)								
Variety		NS	NS					
Hormone		0.82	2.5					
Variety X Hormone		NS	NS					

The explants produced multiple shoots in all the hormone combinations studied (Fig. 2.b). Among the different hormone combinations tried, BAP + NAA (1:1 mgl^{-1}) gave maximum number of shoots in both the varieties. The number of shoots ranged from 2 to 5 with a mean number of 3.8 (var. Jamaica) and 2 to 6 with a mean number of 4.8 (var. Varada) (Table 18) after 45 days of culturing, in this hormone combination.

Most frequently used hormone combination for micropropagation of ginger using vegetative bud was BAP + NAA in different combinations (Sakamura *et al.*, 1986; Inden *et al.*, 1988; Choi and Kim, 1991; Choi, 1991a; Dogra *et al.*, 1994; Huang, 1995; Samsudeen, 1996; Freitez and Casares, 1997; Nirmal Babu, 1997; Sharma and Singh, 1997; Adaniya and Shirai, 2001).

Bhagyalakshmi and Singh (1988) however, reported multiple shoot induction from vegetative buds of ginger in MS medium with 6% sucrose, 20% coconut milk, ascorbic acid at 100 mgl^{-1}, activated charcoal at 250 mgl^{-1} and BA + IBA (0.5:0.4 mgl^{-1}). Vegetative buds were also regenerated on MS medium with BA and IAA in different concentrations with 100 mgl^{-1} adenine sulphate and 3 % sucrose (Palai *et al.*,

1997). Keng and Hing (2004) reported that 9.8 shoots per explant were produced when rhizome buds of ginger was cultured on MS medium with BAP + IBA (2:0.2 Mgl^{-1}).

Though a cytokinin along with one auxin have been reported favorable for multiple shoot induction in ginger, other reports are also there. BAP/ Kinetin alone in different concentrations resulted in successful plant regeneration in ginger (Hosoki and Sagawa, 1977; Balachandran *et al.*, 1990; Oliver, 1996; Pandey *et al.*, 1997; Tyagi *et al.*, 1998; Devi *et al.*, 1999; Jasrai *et al.*, 2000).

4.1.3.3. Number of roots

Analysis of variance for number of roots revealed significant effect of hormones on root development. The varietal effect and variety X hormone interaction for root development were non significant (Table 17).

Mean number of roots observed in the 2 varieties under the different hormone combinations are given in Table 18. The mean number of roots for varieties revealed that the var. Varada produced more number of roots compared to the var. Jamaica. Among the five hormone combinations tried, BAP and NAA (1:1 mgl^{-1}) produced maximum number of roots in both the varieties. The number of roots ranged from 8 to 26 with a mean number of 11.6 in the var. Jamaica and 9 to 21 with a mean number of 15.6 in the var. Varada (Table 18).

Samsudeen (1996) observed that NAA was significantly better over other auxins (IBA or 2,4-D) for root induction from vegetative buds of ginger.

4.1.4. Induction of callus from aerial stem

4.1.4.1. Number of days taken for callus induction

In vitro aerial stem produced callus by second week of culturing in both the varieties. At the same time, the *in planta* aerial stem took 32 days in the var. Jamaica and 25 days in the var. Varada for callus induction.

4.1.4.2. Percentage of explants callused

Percentage of explants callused varied with types of explant, varieties and levels of hormones (Table 19).

Table 19. Effect of explants and varieties on callus induction.

Sl. No	Hormones mgl^{-1} (2,4-D)	Jamaica				Varada			
		In vitro aerial stem	% of callus induced cultures	*In planta* aerial stem	% of callus induced cultures	*In vitro* aerial stem	% of callus induced cultures	*In planta* aerial stem	% of callus induced cultures
1	0.5	+	30	-	-	++	40	-	-
2	1.0	++	70	+	20	+++	60	+	40
3	2.0	+++	90	++	60	+++	100	++	70

+ low callusing; ++ moderate callusing; +++ profuse callusing.

The results revealed that *in vitro* aerial stem gave good result as compared to *in planta* aerial stem, in both the varieties. The callusing response of the var. Varada was better than the var. Jamaica.

In vitro aerial stem of the var. Jamaica produced profuse callusing in MS with 2,4-D (2 mgl^{-1}) and moderate callusing in 2,4-D (1 mgl^{-1}). But the *in planta* aerial stem of this variety showed only moderate callusing with 2,4-D (2 mgl^{-1}). In the var. Varada, *in vitro* aerial stem showed profuse callusing in 2,4-D (1 mgl^{-1} and 2 mgl^{-1}), while the *in planta* aerial stem of the var. Varada produced only moderate callusing in 2,4-D (2 mgl^{-1}) (Table 19).

Choi (1991a) reported callus induction from pseudostem (aerial stem) explants of top, middle or bottom portions with one leaf blade when cultured on the medium containing 0.5 ppm NAA.

Callus induction may vary with the type of explants used. Samsudeen (1996) reported more callusing from the disc portion of the ginger rhizome bud compared to the apex part.

It is generally considered that presence of auxin is required for the induction of callus and 2, 4-D is the most effective and frequently used auxin for callus induction (George, 1993a). In ginger also, an auxin was invariably required for callus induction and 2, 4-D was found most effective (Samsudeen, 1996). However, Kacker *et al.* (1993) compared four auxins viz. IAA, NAA, 2, 4-D and Dicamba and observed that Dicamba was the most effective in inducing callus in ginger. Other workers reported effectiveness of 2, 4-D for callus induction from different explants like leaves (Nirmal Babu *et al.*, 1992 b) and young sprouts (Nadgauda *et al.*, 1980) of ginger. Pu *et al.* (2004) reported that the optimum medium for the induction of ginger callus was MS medium supplemented with 2, 4- D + Kinetin.

4.1.4.3. Color and texture of callus

Eventhough the callus was induced and multiplied in the same medium, the quality and type of the callus produced from different explants varied. Callus induced from *in vitro* aerial stem was hard, nodular and yellowish in colour. The surface of the callus was covered with small hairy structures (hereafter referred to as Type I callus) (Fig. 2.c). *In planta* aerial stem produced soft, sticky callus with pale white or creamy colour, which is friable in nature (hereafter referred to as Type II callus) (Fig. 2.d). Quality of the callus, such as callus color compactness and friability is closely related to the potential of shoot regeneration. The selection of good quality callus during the subculture is important for efficient plantlet regeneration from the callus.

4.1.5. Regeneration of plantlets from callus

4.1.5.1. Number of days taken for regeneration

Type I callus produced roots after 7 days of culturing but no shoot regeneration was observed in both the varieties (Fig. 2.e). Kacker *et al.* (1993) also

observed similar result. Malamug *et al.* (1991) have reported that the organogenic capacity of soft callus of ginger is lost after three subcultures. Lack of shoot regeneration from Type I callus in the present study may be due to non competency of callus. Type II callus showed shoot and root development. Varietal difference was observed in this case. In the var. Jamaica, callus started to produce shoots after 20 days and roots after 23 – 25 days of culturing (Fig. 2.f & g). However, in the var. Varada, it took nearly 30 days in case of shoots and approximately 35 days in case of root regeneration.

4.1.5.2. Number of shoots

Analysis of variance for shoot regeneration revealed significant effect of hormones and variety X hormone interaction on number of shoots. The varietal difference on shoot regeneration was found non significant (Table 20).

Table 20. Analysis of variance for shoot and root regeneration from callus.

Source	Degrees of freedom	No. of shoots			No. of roots		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Variety	1	5.29	5.29	2.64^{ns}	4.00	4.00	0.40^{ns}
Hormone	4	59.54	14.89	7.41**	77.86	19.47	1.96^{ns}
Variety x Hormone	4	73.06	18.27	9.10**	69.70	17.43	1.75^{ns}
Error	90	180.70	2.01		894.80	9.94	
Total	99	318.59			1046.36		

Percentage of shoot regeneration and mean number of shoots regenerated from callus cultures of the two varieties of ginger under different hormone combinations are given in the Table 21. Callus regeneration was observed in all the hormone combinations tried in both the genotypes, but the percentage of regeneration varied slightly with the levels of hormones. In the var. Jamaica the percentage of regeneration varied from 90 to 100% while in the var. Varada 80 to 90% (Table 21). Maximum regeneration percentage was observed in the combination of BAP + NAA

(1:1, 2:0.5 & 5:0.5 mgl^{-1}) in the case of the var. Jamaica while BAP + NAA (1:0.5 and 2:0.5 mgl^{-1}) gave 100 percent regeneration in the var. Varada.

Table 21. *In vitro* shoot and root regeneration from callus on MS medium fortified with different growth hormones.

Growth regulators in basal medium (mgl^{-1})	Jamaica			Varada			Mean	
BAP : NAA	% of shoot inducing culture	No. of shoots	No. of roots	% of shoot inducing culture	No. of shoots	No. of roots	Shoot	Root
1.0 : 0.5	90	1.9	4.6	100	1.9	5.9	1.9	5.3
1.0 : 1.0	100	5.8	8.3	80	2.1	5.5	4.0	6.9
2.0 : 1.0	90	2.2	5.1	80	2.1	6.5	2.2	5.8
2.0 : 0.5	100	2.7	6.4	100	4.1	6.3	3.4	6.4
5.0 : 0.5	100	2.5	8.7	80	2.6	6.9	2.6	7.8
Mean		3.0	6.6		2.6	6.2		
CD (P=0.05)								
Variety		NS	NS					
Hormone		0.90	NS					
Variety X Hormone		1.3	NS					

Mean number of shoots for varieties showed that the var. Jamaica produced more number of shoots as compared to the var. Varada though the varietal difference was non significant. The var. Jamaica produced maximum number of shoots with BAP and NAA (1:1 mgl^{-1}). The number of shoots ranged from 3 to 12 with a mean number of 5.8. In the var. Varada the optimum concentration for shoot regeneration was BAP and NAA (2:0.5 mgl^{-1}). The number of shoots ranged from 2 to 6 with a mean number of shoots 4.1 (Table 21) (Fig. 2.h & i).

Successful plantlet regeneration from aerial stem (pseudostem) derived calli were reported by Choi (1991a). High plantlet regeneration was observed from the

callus induced from the base or middle portion explants when cultured on the medium containing 0.1-1.0 ppm NAA and1.0 ppm BA.

Kacker *et al.* (1993) reported successful callus regeneration of ginger in medium containing BAP alone. Plantlet formation in ginger callus was observed with BAP in high concentration and 2, 4-D in low concentration (Samsudeen, 1996; Nirmal Babu, 1992 b). Pu *et al.* (2004) reported that the addition of 2.0 mgl^{-1} Kinetin + 0.5 mgl^{-1} NAA favoured the regeneration of plantlets from ginger callus.

4.1.5.3. Number of roots

Analysis of variance for root regeneration revealed non significant effect of variety, hormones and variety X hormone interaction for root development (Table 20).

Mean number of roots regenerated from callus cultures of the two varieties of ginger under different hormone combinations are given in the Table 21. The results showed that both the varieties produced almost same number of roots. However, the rate of rhizogenesis was more in BAP and NAA (5.0:0.5 mgl^{-1}) for both the varieties. The number of roots ranged from 5 to 16 with a mean number of 8.7 in the var. Jamaica and 2 to 18 with a mean number of 6.9 in the var. Varada (Table 21).

4.1.6. Induction of somatic embryos

4.1.6.1. Number of days taken for somatic embryo induction

Varietal difference was observed in ginger for the somatic embryo induction. Type II callus started to produce somatic embryos after 30-35 days of culturing in the var. Jamaica, while it took nearly 45 days in case of the var. Varada.

4.1.6.2. Response of callus cultures

Type II calli showed callus proliferation in all cultures containing 2,4-D and BAP in different concentrations, in both the varieties. However, percentage of somatic embryo induction varied with different varieties. In the var. Jamaica, 100 % cultures produced somatic embryos and in the var. Varada, only 50 % cultures resulted in somatic embryo induction.

In both the varieties, Type I callus did not turn to embryogenic but produced roots in all the cultures. However, the Type II callus showed proliferation in all the combinations tried and 2,4-D + BAP (1: 0.5 mgl^{-1}) were found optimum for callus proliferation in the var. Jamaica and 2,4-D + BAP (1.0 : 1.0 mgl^{-1}) in the var. Varada.

The Type II calli were subjected to stress for 40 to 60 days without subculturing. The desiccated calli started to produce good quality, white friable callus (Fig. 3.a). This calli were then subcultured to the medium containing BAP alone in different concentrations for somatic embryo induction.

Morphogenic responses were observed from the calli, cultured on the medium containing BAP only ($2mgl^{-1}$). These calli turned embryogenic by the first subculturing itself in the var. Jamaica and by the second subculturing in the var. Varada. Hua *et al.* (2005) also reported varietal variation for somatic embryogenesis in ginger.

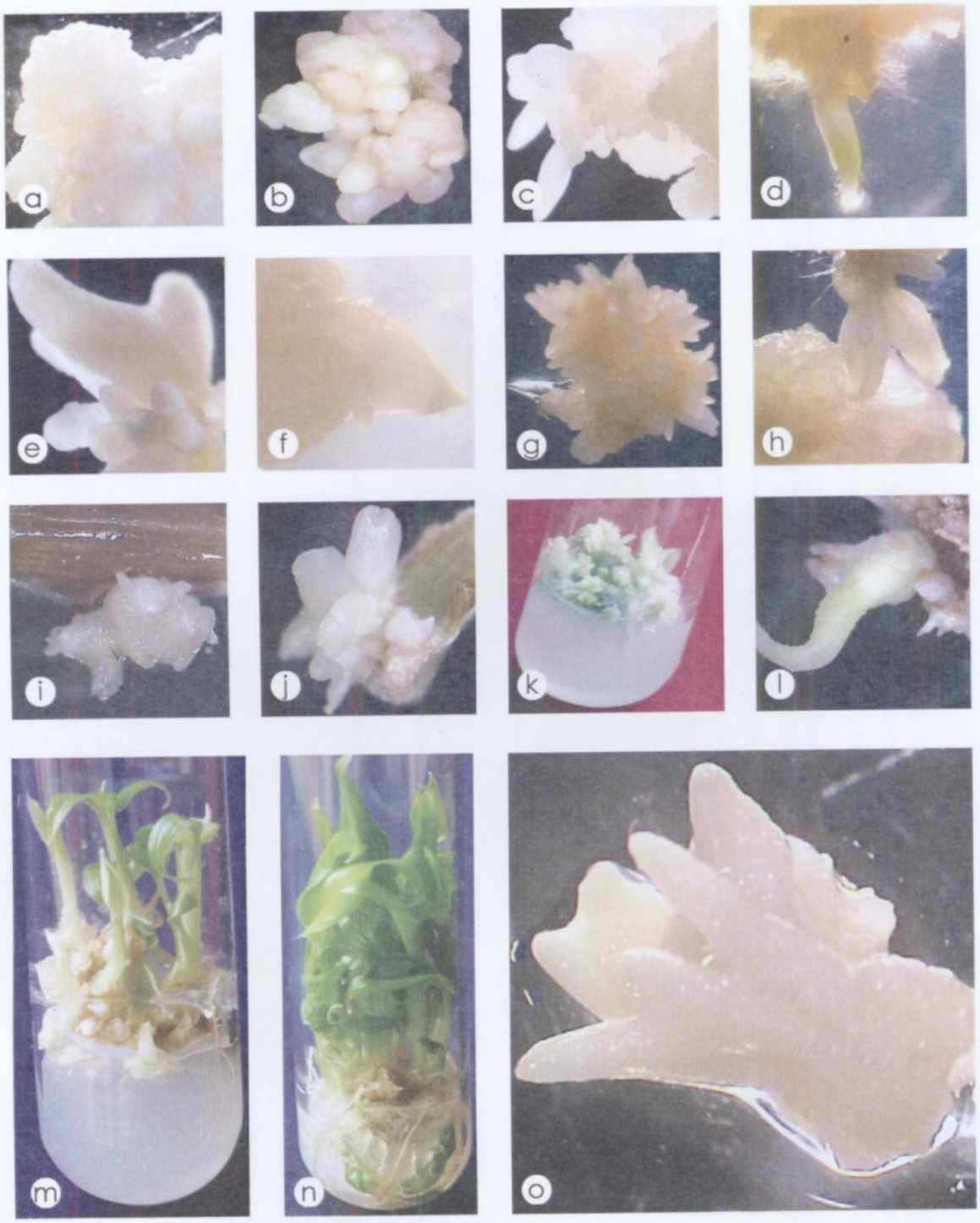


Fig. 3. a). White friable callus induced from desiccated calli. b). White globular embryoids on the peripheral region of the callus. c). Globular and oval shaped embryos. d & e). Club shaped embryo with cotyledon and scutellum initiation. f). Somatic embryo with green coloured coleoptile. g & h). Secondary somatic embryogenesis. i). *In planta* aerial stem explant of var. Jamaica showing direct somatic embryo induction (0.5 TDZ + 1.0 IBA mg l^{-1}). j). Leaf base explant of var. Varada showing direct somatic embryo induction (0.5 TDZ mgl^{-1}). k). Mature embryos showed green color after 12 days of culturing (1.0 BAP + 1.0 NAA mgl^{-1}). l). Closeup view of somatic embryo germination. m & n). Shoot and root proliferation from somatic embryos cultured on 2 BAP + 0.2 NAA mgl^{-1} (var. Jamaica) and 2 BAP + 1 NAA mgl^{-1} (var. Varada), respectively. o). Irregular and aberrant formation of somatic embryos.

Source of explants is considered to be an important factor in the induction of somatic embryos. Significant differences in morphogenetic potential were also observed within a single explant. The terminal hypocotyl segments of eggplant showed better embryogenic potential than the median segments (Sharma and Rajam, 1995a, b). Similar position effect on somatic embryogenesis was also observed in pepper (Kintzios *et al.*, 2000).

Borkind *et al.* (1988) reported that the removal of 2,4-D from the medium facilitated the progression of pro embryonic cells to the advanced stages of somatic embryos. Vincent *et al.* (1992) also observed that the replacement of 2,4-D with BAP in the culture medium produced globular embryos in *Kaempferia galanga* L.

Zimmerman (1993) reported that new gene products are synthesized upon the removal of 2,4-D, which are required for the transition from globular stage to the heart shaped structure. When 2,4-D was used in low concentration or totally eliminated from the media, the embryos were promoted to become mature and started differentiation in ginger (Hua *et al.*, 2005).

The embryogenic calli produced localized groups of cells which differentiated into embryogenic forms on the peripheral region of the callus and latter differentiated into white globular embryoids (Fig. 3.b) after 30 days of culture. This process continued in the subsequent subculturing in the same medium. The spherical/globular embryo developed into oval shaped embryo (Fig. 3.c). After 45 days, the somatic embryos differentiated into a more mature, elongated club shaped or cylindrical stage and cotyledon initiation as well as signs of scutellum initiation occurred at this stage (Fig. 3.d & e). After 60 days the coleoptile became green and the shoot got completely differentiated with one or two leaf plumules (Fig.3.f). At this stage, the embryos started germinating and developing shoot.

Shah (1982) reported that the monocotyledons embryo is cylindrical and shoe shaped with a slightly pointed distal end and broad blunt coleorhizal end.

Thus the somatic embryos in ginger followed typical stages described for other monocot systems: globular embryo stage, club shaped cotyledon initiation stage and the plumules initiation stage. Details of monocot embryo systems were reported early (Vasil *et al.*, 1984; Guiderdoni and Demarly, 1988; Smith and Krikorian, 1991).

4.1.7. Secondary somatic embryogenesis

Secondary somatic embryogenesis was also observed when the embryogenic calli with somatic embryos were transferred to fresh medium with or without growth hormones (BAP – 0.5, 1.0, 2.0 mgl^{-1}) (Fig. 3. g & h). The presence of BAP hastened the secondary somatic embryo induction. Secondary somatic embryos may have their origin from the proliferation of cell in the developed somatic embryos.

4.1.8. Induction of direct somatic embryos

In few explants direct somatic embryogenesis was observed.

4.1.8.1. Number of days taken for somatic embryo induction

In planta aerial stem explants took approximately 30 to 40 days to induce somatic embryos and leaf explants took 45 days for direct somatic embryo induction.

4.1.8.2. Percentage of culture responded

Low percentage of response was observed in this case. Only 30% of cultures responded in direct induction of somatic embryos in the var. Jamaica and 20 % in the var. Varada.

4.1.8.3. Response of explants

Varietal difference was observed in ginger for direct somatic embryo induction. *In planta* aerial stem explants of the var. Jamaica produced somatic embryos directly from the explants in the cultures containing TDZ + IBA (0.5 + 1.0 mg l^{-1}) (Fig. 3. i). In the var. Varada, direct somatic embryos were induced from the

leaf base cultured on the medium containing TDZ (0.5 mgl^{-1}) (Fig. 3. j). No plantlet regeneration was observed in the direct regenerated somatic embryos.

4.1.9. Germination of somatic embryos

4.1.9.1. Number of days taken for regeneration

The germination of embryos was obtained by second week of culturing. The mature embryos showed green color after 12 days of culturing in the regeneration medium containing BAP and NAA in different combinations (Fig. 3. k & l). Then these embryos produced shoots and roots simultaneously.

4.1.9.2. Number of shoots

Varietal influence was not significant for shoot regeneration from somatic embryos but the effects of hormones and hormone X variety interaction were found highly significant (Table 22).

Table 22. Analysis of variance for shoot and root regeneration from somatic embryo.

Source	Degrees of freedom	No. of shoots			No. of roots		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Variety	1	0.18	0.18	0.06 ^{ns}	5.78	5.78	0.48 ^{ns}
Hormone	4	162.88	40.72	14.39**	73.48	18.37	1.54 ^{ns}
Variety x Hormone	4	223.52	55.88	19.75**	118.92	29.73	2.49 ^{ns}
Error	40	113.20	2.83		477.60	11.94	
Total	49	499.78			675.78		

Percentage of somatic embryo germination and mean number of shoots regenerated from somatic embryos of the two varieties of ginger under different hormone combinations are given in the Table 23.

Germination of somatic embryos was observed in all the hormone combinations tried and the percentage of germination varied from 60 to 100% in both the varieties. Maximum percentage of shoot regeneration was observed in the

medium containing BAP and NAA (0.2: 0.2; 1.0: 1.0; 2.0: 02) in the var. Jamaica and BAP and NAA (1.0:0.5; 2.0: 0.2; 2.0: 1.0) in the var. Varada (Table 23).

Table 23. *In vitro* shoot and root regeneration from somatic embryos on MS medium fortified with different growth hormones.

Growth regulators in basal medium (mgl^{-1})	Jamaica			Varada			Mean	
BAP : NAA	% of shoot inducing culture	No. of Shoot	No. of Root	% of shoot inducing culture	No. of shoots	No. of roots	Shoot	Root
0.2 : 0.2	100	4.4	6.4	60	1.4	8.2	2.9	7.3
1.0 : 0.5	60	0.8	12.6	100	2.2	7.6	1.5	10.1
1.0 : 1.0	100	1.8	9.0	80	1.2	10.2	1.5	9.6
2.0 : 0.2	100	8.2	9.0	100	2.8	5.2	5.5	7.1
2.0 : 1.0	80	2.0	7.8	100	9.0	10.2	5.5	9.0
Mean		3.4	9.0		3.3	8.3		
CD (P=0.05)								
Variety		NS	NS					
Hormone		0.74	NS					
Variety X Hormone		1.04	NS					

Among the five hormone combinations tried, the optimum concentration for shoot regeneration was 2 mgl^{-1} BAP + 0.2 mgl^{-1} NAA (var. Jamaica) and 2 mgl^{-1} BAP + 1 mgl^{-1} NAA (var. Varada). The number of shoots ranged from 5 to 12 with a mean number of 8.2 in the var. Jamaica and 5 to 13 with a mean number of 9.0 in the var. Varada (Table 23) (Fig. 3. m & n). Hua *et al.*, (2005) reported that BAP was effective for regeneration of somatic embryos also, besides induction of somatic embryos.

Kacker *et al.* (1993) also reported that inclusion of BAP (2.2-6.7 µM) in the regeneration medium hastened the germination of somatic embryos.

It was observed that some well developed somatic embryos (20-30%) failed to germinate and formed irregular and aberrant structures (Fig. 3. o). Embryogenic

callus yielded various 'neo morphs-like' structures (developmentally abnormal or poorly developed pseudo- embryonic forms that did not yield plantlets and eventually died) comprising of a root and a poorly developed or altogether lacking shoot tip meristem in Day lily (Smith and Krikorian, 1991).

Germination without plantlet recovery, i.e. the formation of root but not an epicotyl, may be due to malformation of the apical shoot meristem or the embryos being damaged during its isolation for transfer to germination medium (Valladares *et al.*, 2006).

4.1.9.3. Number of roots

Effects of varieties, hormones and variety X hormone interaction were not significant for root regeneration (Table 22).

Mean number of roots regenerated from somatic embryos of ginger under different hormone combinations are given in the Table 23. However, maximum root regeneration was observed in BAP and NAA (1:0.5 mgl^{-1}) in the var. Jamaica and BAP and NAA (1:1 and 2:1 mgl^{-1}) in the var. Varada. The number of roots ranged from 7 to 21 (mean 12.6) in the var. Jamaica and 5 to 15 (mean 10.2) in the var. Varada (Table 23).

4.1.10. Shoot proliferation through subculturing

4.1.10.1. *In planta* aerial stem derived plantlets

The shoots obtained from the TDZ treatments were very small and abnormal, which have been attributed to the detrimental effects of the TDZ. Long exposure of explants to TDZ inhibited the capacity of shoot multiplication. TDZ is known to inhibit shoot proliferation especially in the solid cultures (Amutha *et al.*, 2006). Therefore shoot multiplication can be manipulated by altering the type and concentration of hormones in the culturing medium. Thus, the cultures were not maintained for long in the same medium. The inhibition of shoot elongation may be

due to the high cytokinin activity of TDZ. Cytokinins commonly stimulate shoot proliferation while inhibiting their elongation (Huetteman and Preece, 1993).

In planta explants subcultured onto the medium containing different concentrations of BAP + NAA took approximately 45 days to show first signs of shoot proliferation. The explants showed low rate of proliferation also. Hence to hasten the shoot proliferation, the *in planta* explants with shoot and root initials were subcultured onto liquid medium supplemented with different concentrations TDZ + IBA and BAP + NAA. The explants cultured in the medium containing TDZ + IBA (1.0:0.5 and 1.0:1.0 mgl^{-1}) started proliferation after 7 days of culturing. The rest of the combinations had no effect on shoot proliferation. After 20 to 25 days the shoots multiplied and formed a clump. The length of the shoot buds increased considerably but turned white in colour.

The subculture in the liquid medium supplemented with TDZ or BAP, increased the organogenic potential and it helps to improve the regeneration efficiency in *Centaurium erythrace* Rafn and *Phaseolus vulgaris* L. (Piatczak *et al.*, 2005; Veltcheva and Svetleva, 2005). According to Mohamed *et al.* (2006) the liquid culture changes the physiological nature of explants. The explants are completely submerged in the liquid medium, where they apparently uptake nutrients and growth hormones that favour the development of organogenesis.

For further shoot and root proliferation, the excised explants with shoot and roots were transferred onto MS solid medium with BAP and NAA (1:1 mgl^{-1}). These shoot turned green in colour after 12 days of culturing. Well developed plantlets could be obtained by 45 days of culturing in this medium. Profuse rooting was also observed.

4.1.10.2. *In vitro* aerial stem, vegetative bud, callus and somatic embryo derived plantlets

The explants with shoot and root initials were cut into small pieces and subcultured onto MS medium with BAP and NAA (1:1 mgl^{-1}). Shoot and root proliferation were observed in these explants after 7 days of subculturing and well developed plantlets could be established (Fig. 2. j).

The number of shoots and roots increased with subculturing. Chithra *et al.* (2005) reported that the number of roots exhibited a positive correlation with subcultures.

4.2. Histological studies

4.2.1. Ontogeny of shoot/root initials from in planta *aerial stem*

The longitudinal sections of *in vitro* and *in planta* aerial stem revealed the presence of two distinct regions, inner zone and outer zone (Lincy *et al.*, 2004). These two zones contain numerous meristamatic regions called primary thickening meristem (PTM) (Fig. 4. a). Gifford and Bayer (1995) reported that PTM may be responsible for the stem thickening and production of shoots and roots in monocotyledons. Tomlinson (1969) reported that aerial stem of monocotyledons consists of nodes and internodes. In the present study, it was observed that the aerial stem of ginger also consist nodes and internodes.

The longitudinal and transverse sections of the multiple shoots induced aerial stem showed that the shoots regenerated from apical meristem and PTM of the explant (Fig. 4. b & c). The root primordia were regenerated from the cells present in the PTM.

The first change i.e., the onset of active cell division of PTM/apical meristem observed after 5-10 days on regeneration medium. Continuous meristamatic activity in these cells resulted in the formation of adventitious shoot primordia that ruptured

the explant surface. Development of adventitious shoot primordial continued under the influence of cytokinin activity and produced multiple shoots and resulted in a rosette appearance. Similar result was observed in *Lapageria rosea* (Ruiz et Pav.) (Mc Kinless and Alderson, 1991).

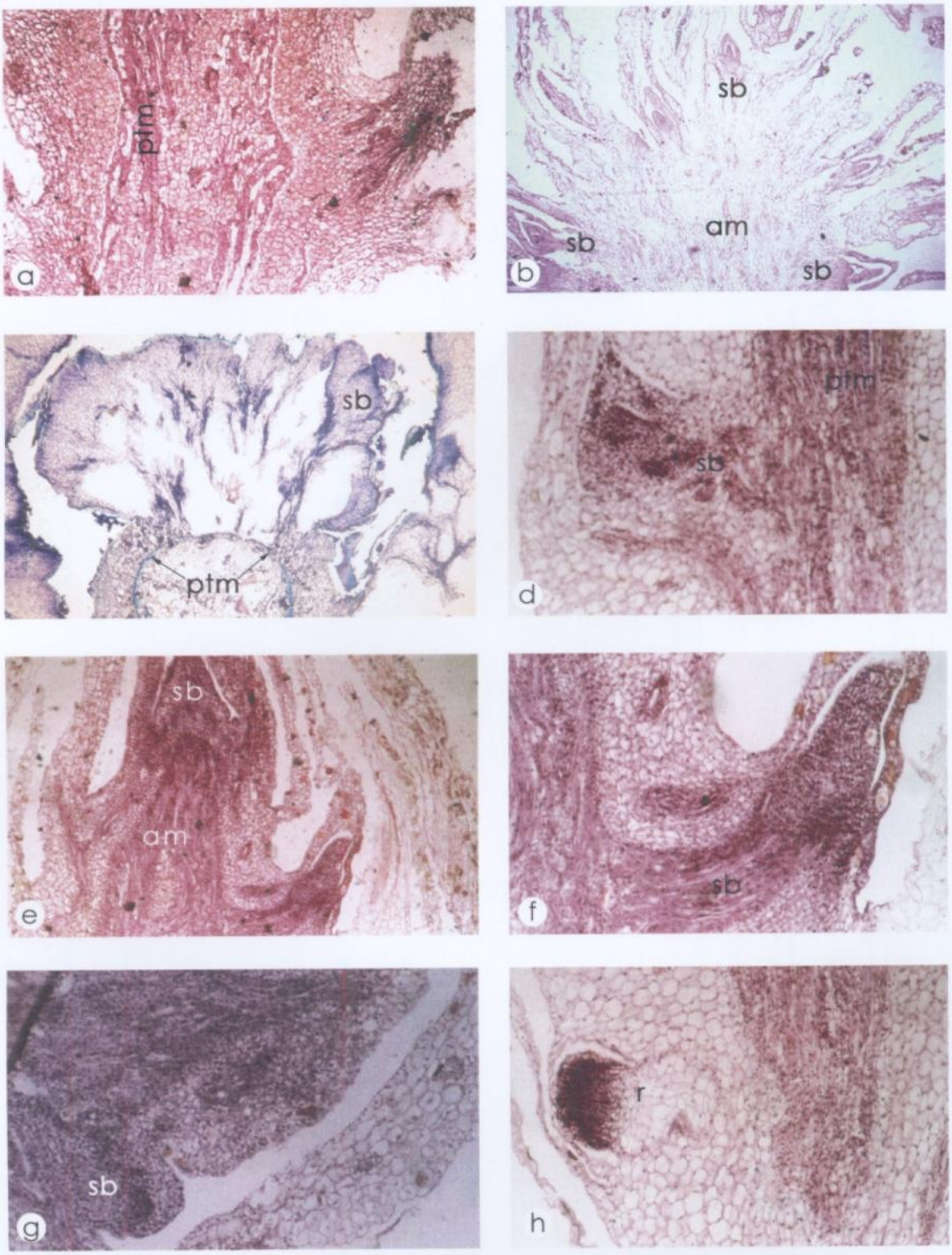


Fig. 4. a). Longitudinal section of *in planta* aerial stem showing primary thickening meristem (PTM). b). Multiple shoot regeneration from apical meristem of *in planta* aerial stem. c). Shoot regeneration from primary thickening meristem of *in planta* aerial stem. d). Differentiation of PTM of *in vitro* aerial stem in to shoot. e). Shoot bud initiation from apical meristem of *in vitro* aerial stem. f). Shoot development from axillary meristem of *in vitro* aerial stem. g). Shizogenesis from accessory bud. h). Differentiation of root primordial from PTM of *in vitro* aerial stem.

am - Apical meristem, ptm - Primary thickening meristem, r - Root, sb - Shoot bud

4.2.2. *Ontogeny of shoot/root initials from* in vitro *aerial stem*

In immature aerial stem, the nodes are constricted and each node carries an axillary bud and each axillary bud has its own accessory buds.

The transverse and longitudinal sections of the multiple shoot induced *in vitro* aerial stem showed four types of differentiations.

i) The differentiation of PTM cells into shoots (Fig. 4. d).

ii) From the apical meristem (Fig. 4. e)

iii) The dormant axillary bud present in the nodal region of the aerial stem activates due to the hormonal action, leading to shoot formation (Fig. 4. f).

iv) The accessory buds present in the axillary bud also emerge out and produces shoot leading to multiple shoot induction (Fig. 4. g).

The root primordial got differentiated endogenously from the cells present in the endodermoid layer or PTM (Fig. 4. h).

In vitro adventitious shoot development from aerial stem of ginger and ontogeny of shoot/root were reported by Lincy *et al.* (2004). Mc Kinless and Alderson (1991) reported the similar results in *L. rosea,* a rhizomatous plant. Anatomical study of *in vitro* shoot cultures of *L. rosea* revealed the presence of axillary buds in the leaf axis of the aerial stem and presence of vascular traces. Adventitious shoot formation from the leaf axillary buds of aerial stem of stressed field grown ginger plant is also reported (Sasikumar, 2005).

4.2.3. *Somatic embryo induction / origin of somatic embryo*

Anatomical sections prepared from the embryogenic cultures of ginger containing somatic embryos at different developmental stages revealed the ontogeny of somatic embryos. The histological sections showed groups of meristamatic cells formed at the peripheral region of embryogenic callus, which can be clearly identified from the normal non embryogenic calli (Fig. 5. a & b). These meristamatic cells were

small compact and densely cytoplasmic with distinct nuclei. The embryos developed from single cells, which underwent continuous divisions and formed two, four, six and eight celled stages (Fig. 5. c, d & e). After that, it formed a globular mass (proembryoids) that showed a distinct epidermis (Fig. 5. f). Further differentiation of theses structures lead to the formation of globular, oval shaped embryos on the surface of the callus (Fig. 5. g & h). The embryo was attached to the embryogenic callus with a prominent multicellular stalk, the suspensor (Fig. 5. i).

Swamy (1982) observed that the quadrant and octant stages are invariable and necessary stages in the embryogenesis of both dicotyledons and monocotyledons. Kacker *et al.* (1993) observed the presence of nodular structures containing richly cytoplasmic cells delimited by a single layered epidermis. These authors also observed stalked somatic embryos with scutellum, coleoptile and coleorhiza in ginger.

At the cylindrical stage of somatic embryo, the embryo primordium started to grow as a broad outgrowth and a notch arose at its terminal region of the embryo (Fig. 5. j & k). The scutellum developed as a hump opposite the notch where the shoot apex is organized and the coleoptile developed as a circular primordium around the shoot apex. In ginger, shoot apex had a lateral position and the root apex developed opposite the shoot apex. Thus the somatic embryos had a scutellum, coleoptile, shoot apex and coleorhiza with an independent vascular system that is not connected to the maternal tissue (Fig. 5. l & 6. a).

The primary and secondary somatic embryos at the peripheral region of the callus formed a bunch like appearance (Fig. 6. b). Secondary somatic embryos were developed due to the proliferation of cells in the coleorhizal part of mature primary somatic embryos (Fig. 6. c).

Somatic embryos were induced directly from the primary thickening meristem of *in planta* aerial stem explant and from the epidermal tissues of leaf base explant (Fig. 6. d & e) with distinct scutellum, coleoptile, shoot apex and root apex (Fig. 6. f).

Wardlaw (1955) reported that in monocotyledonous embryo, the shoot apex occupies a lateral position in the somewhat cylindrical embryo and the cotyledon is terminal. Bechtel and Pomeranz (1978) and Batygina (1969) observed presence of scutellum and coleoptile in the monocot embryo.

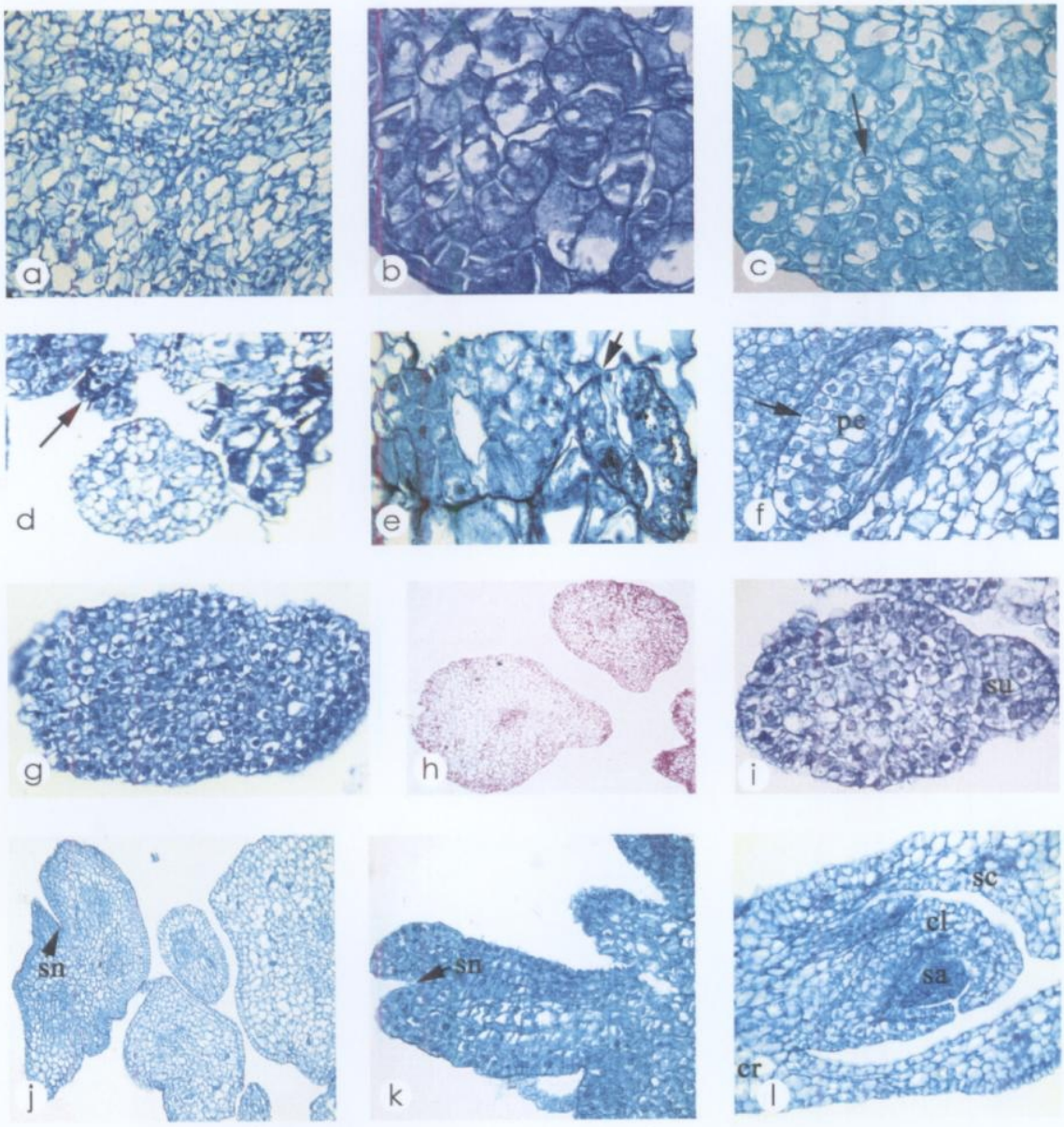


Fig. 5. a). Normal, non embryogenic calli. b). Groups of meristamatic cells at the periferal region of embryogenic callus. c, d & e). Two, six and eight celled stages of embryogenesis (arrows). f). Globular mass (proembryoid) with a distinct epidermis. g & h). Globular embryos on the surface of the callus. i). Globular embryo with multicellular suspensor. j & k). Scutellar notch at the terminal region of the embryo. l). Somatic embryo with scutellum, coleoptile, shoot apex and coleorhiza.

cl - Coleoptile, cr- Coleorhiza, pe -Proembryoid, sa - Shoot apex, sc - Scutellum, sn - Scutellar notch, su - Suspensor

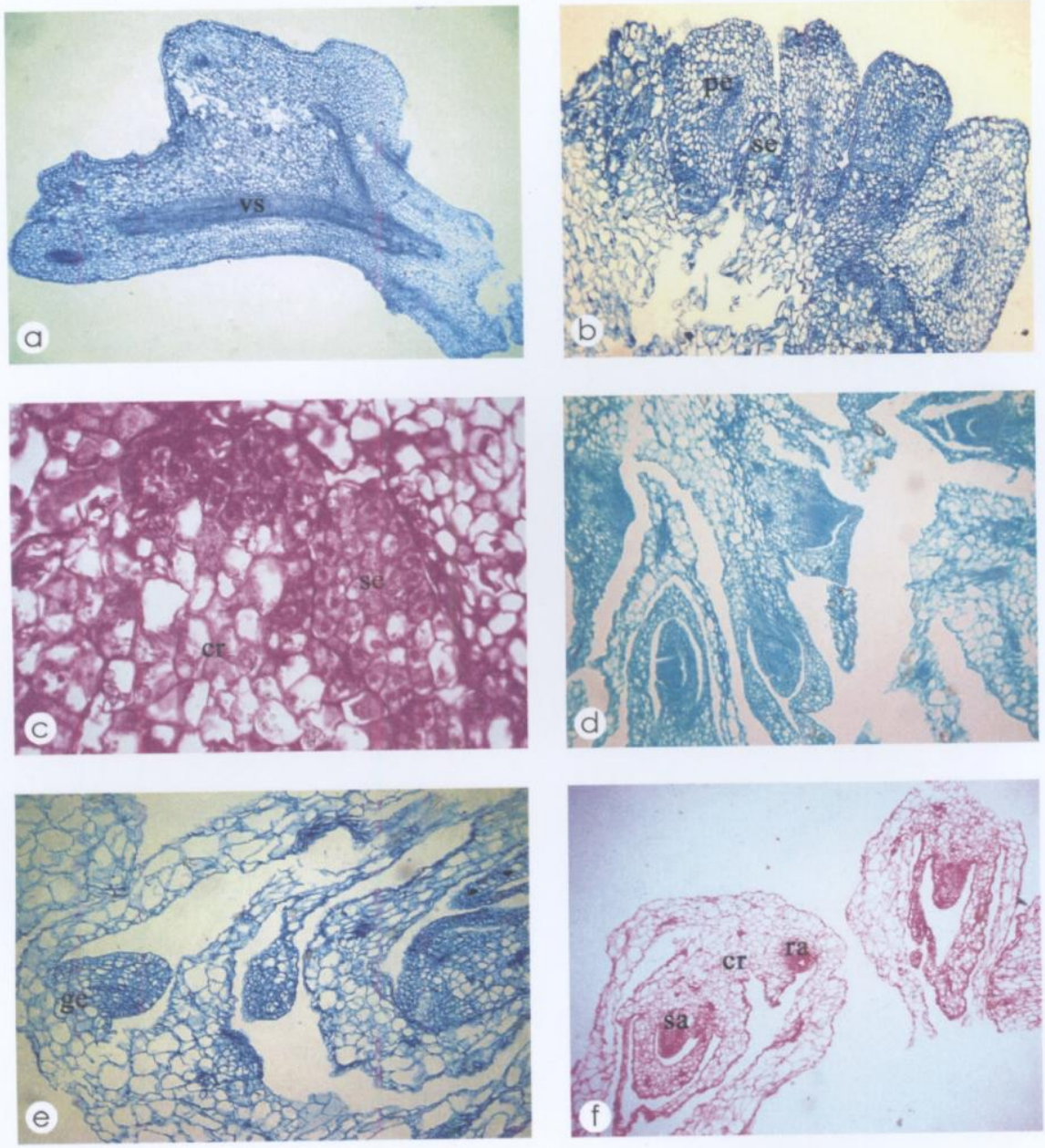


Fig. 6. a). Somatic embryo with an independent vascular system. b). Primary and secondary somatic embryos. c). Secondary somatic embryos originating from the coleorhizal part of primary somatic embryo. d). Direct somatic embryo induction from aerial stem explant. e). Direct somatic embryo induction from leaf explant - globular stage. f). Single somatic embryo with shoot and root apex.

cr - Coleorhiza, ge - Globular embryo,pe - Primary embryo, ra - Root apex, sa - Shoot apex, se - Secondary embryo, vs - Vascular system.

4.3. Genetic fidelity analysis

Genomic DNA was isolated from young leaves of ginger plants using CTAB method. The DNA was collected as pelletted samples. High molecular weight, high quality DNA was obtained from the tissues. The yield of DNA varied from 120 – 180 µg /g tissue.

4.3.1. RAPD profiles of ginger using different primers

Out of the 25 different decamers tested, 18 primers (9 OPB, 5 OPJ and 4 OPD) produced good amplification products.

4.3.1.1. Aerial stem regenerated plants

No polymorphism could be observed in the RAPD profile of aerial stem regenerated ginger plants. The details of bands produced in the RAPD profile of the two varieties studied are given in the Table 24.

Table 24. The details of number of bands produced in the RAPD profile of the two ginger varieties.

Sl. No	Primer	Sequence (5'-3')	var. Jamaica						var. Varada					
			Total no. of bands	Size of amplified products (bp)	Mono mor. bands	Poly mor. bands	Size of poly mor. bands (bp)	% of poly morphysm	Total no. of bands	Size of amplified products (bp)	Mono mor. bands	Poly mor. bands	Size of poly mor. bands (bp)	% of poly morphysm
1	OPB 01	GTTTCGCTCC	7	1750-350	7	0	-	-	8	1400-350	8	0	-	-
2	OPB 06	TGCTCTGCCC	10	1850-500	10	0	-	-	9	1850-500	9	0	-	-
3	OPB 07	GGTGACGCAG	7	1700-300	7	0	-	-	6	2200-500	5	1	2200	16.7 %
4	OPB 09	TGGGGGACTC	7	1300-250	7	0	-	-	8	1300-300	8	0	-	-
5	OPB 10	CTGCTGGGAC	6	1900-650	5	1	1250	16.7 %	8	1900-700	8	0	-	-
6	OPB 11	GTAGACCCGT	6	2000-250	6	0	-	-	5	2000-250	3	2	1900&1800	40 %
7	OPB 14	TCCGCTCTGG	7	1900-400	7	0	-	-	7	1900-400	7	0	-	-
8	OPB 15	GGAGGGTGTT	11	1500-250	11	0	-	-	10	1900-250	6	4	1800,1400,600&500	40 %
9	OPB 17	AGGGAACGAG	6	2100-750	6	0	-	-	10	2100-250	10	0	-	-
10	OPD 02	GGACCCAACC	10	1750-250	10	0	-	-	10	1750-250	10	0	-	-
11	OPD 03	GTCGCCGTCA	8	1800-300	8	0	-	-	6	1600-250	6	0	-	-
12	OPD 11	AGCGCCATTG	10	1800-250	10	0	-	-	10	1800-250	10	0	-	-
13	OPD 13	GGGGTGACGA	10	2000-400	8	2	2200,1400	20 %	10	2200-200	10	0	-	-
14	OPJ 01	CCCGGCATAA	6	1300-250	6	0	-	-	5	2000-500	2	3	2000,1200&600	60 %
15	OPJ 06	TCGTTCCGCA	5	1900-700	5	0	-	-	6	1800-750	6	0	-	-
16	OPJ 07	CCTCTCGACA	10	2000-500	10	0	-	-	7	2000-300	3	4	1000,850,750&500	57 %
17	OPJ 10	AAGCCCGAGG	7	1900-300	7	0	-	-	7	1700-300	7	0	-	-
18	OPJ 16	CTGCTTAGGG	4	2000-500	4	0	-	-	4	2000-500	4	0	-	-

Primer OPB 1 gave good amplification products that were monomorphic across all the samples of the two varieties. The banding pattern showed 7 DNA fragments (1750-350 bp) in the var. Jamaica (Fig. 7. a) and 8 amplicons (1400 – 350 bp) in the var. Varada (Fig.7. e). OPB 6 primer produced 10 monomorphic bands in the var. Jamaica (Fig. 7. b) and 9 monomorphic bands in the var. Varada (Fig. 7. f). The size of bands ranged from 1850 – 500 bp in both the varieties. RAPD profile of primer OPB 7 showed 7 bands, which were monomorphic across all the samples, with size ranging from 1700 to 300 bp in the var. Jamaica (Fig. 7. c). However, in the var. Varada, 6 monomorphic bands of size 2200 to 500 bp were observed (Fig. 7. g) (Table 24).

In the case of OPB 9, the primer produced 7 monomorphic bands (1300 to 250 bp) in the var. Jamaica and 8 monomorphic bands (1300 to 300 bp) in the var. Varada. The primer OPB 10 produced 6 bands with the size of 1900 to 650 bp in the case of the var. Jamaica (Fig. 7. d) and 8 bands with size ranging from 1900 to 700 bp in the var. Varada (Fig.7. h). The bands were monomorphic across all the samples of both the varieties. Six DNA fragments in the var. Jamaica and 5 amplicons in the var. Varada (Fig. 8. d) were observed in the RAPD profile of primer OPB 11. The size of amplicons ranged from 2000 to 250 bp in both the varieties (Table 24).

RAPD profile of aerial stem regenerated plantlets showed 7 monomorphic bands with size ranging from 1900 to 400 bp in the var. Jamaica (Fig. 8. a.) and var. Varada in the case of primer OPB 14. The primer OPB 15 produced 11 monomorphic (1500 to 250 bp) in the var. Jamaica (Fig. 8. b) and 10 monomorphic bands (1900 to 250 bp) in the var. Varada (Fig. 8. e). In case of OPB 17, the bands were monomorphic across all the samples of both the varieties. In the var. Jamaica, 6 bands with the size of 2100 to 750 bp (Fig. 8. c) and in the var. Varada, 10 bands with the size of 2100 to 250 bp were observed (Fig. 8. f) (Table 24).

RAPD profiles of aerial stem regenerated plantlets of the var. Jamaica and the var. Varada showed 10 monomorphic bands (1750 to 250 bp) in case of OPD 2 primer (Fig. 9. a & e). OPD 3 primer produced 8 DNA fragments (1800 to 300 bp) in the var. Jamaica (Fig. 9. b) and 6 DNA fragments (1600 to 250 bp) in the var. Varada (Fig. 9. f). RAPD profile of primer OPD 11 produced 10 monomorphic bands with the size rang of 1800 to 250 bp in both the varieties (Fig. 9. c & g). Primer OPD 13 produced 10 monomorphic bands across all the samples of the var. Jamaica (Fig. 9. d) and the var. Varada with the size ranged from 2000 to 400 and 2200 to 300 bp, respectively (Fig. 9 .h) (Table 24).

The OPJ series primers produced monomorphic DNA fragments across the samples in both the varieties. The primer OPJ 1 produced 6 bands with size ranging from 1300 to 250 bp in the var. Jamaica (Fig. 10. a) and 5 bands with the size rang of 2000 to 500 bp in the var. Varada (Fig. 10. d). RAPD profile of aerial stem regenerated plantlets showed 5 bands (1900 to 700 bp) in the var. Jamaica and 6 bands (1800 to 750 bp) in the var. Varada (primer OPJ 6). The RAPD profile of primer OPJ 7 showed 10 DNA fragments with the size of 2000 to 500 bp in the var. Jamaica (Fig. 10. b) and 7 DNA fragments with the size ranged from 2000 to 300 bp in the var. Varada (Fig. 10. e). In case of primer OPJ 10, the var. Jamaica and the var. Varada produced 7 bands with size ranging from 1900 to 300 bp and 1700 to 300 bp, respectively (Fig. 10. c & f). But in the case of OPJ 16 only 4 bands were obtained in both the varieties with size ranging from 2000 to 500 bp (Table 24).

4.3.1.2. Vegetative bud regenerated plants

No polymorphism was observed in the RAPD profile of vegetative bud regenerated plants. The banding pattern was akin to that observed in the aerial stem regenerated plants (under section 4.4.1.1.).

The RAPD primers used in the present study failed to detect polymorphism in the aerial stem and vegetative bud regenerated plantlets, though they showed some

morphological differences (tiller number, plant height etc.). Rout *et al.* (1998) reported genetic stability of micropropagated ginger plants using RAPD markers. All RAPD profiles of the micropropagated plants were monomorphic and similar to those of field grown control plants. Salvi *et al.* (2002) reported genetic stability of micropropagated turmeric plants using RAPD markers.

Micropropagated plants from the cultures of preformed structures such as shoot tip and axillary buds have been reported to maintain clonal fidelity (Wang and Charles, 1991; Ostry *et al.*, 1994). Shenoy and Vasil (1992) reported that micropropagation through meristem culture is generally associated with low risk of genetic instability because the organized meristem are generally more resistant to genetic changes that might occur during cell division or differentiation under *in vitro* conditions.

In the present study too, the amplified products of aerial stem and vegetative bud regenerated plants exhibited monomorphism among all the samples and were similar to those from control plants (true to type).

The number of bands produced by the different primers showed variations in the present study. Variation in number of monomorphic bands in micropropagated plants using different primers has been reported earlier workers (Potter and Jons, 1991; Rani *et al.*, 1995; Angel *et al.*, 1996; Rout *et al.*, 1998).

Though some morphological variation observed among the tissue cultured plants regenerated from different explant sources, the molecular analysis of aerial stem and vegetative bud regenerated plants did not reveal polymorphism. The lack of correspondence between molecular markers and morphological features is already known. Arunachalam *et al.* (2005) reported that the morphological variation due to the physical environment may not be detectable by RAPD analysis.

However contradictory observations of tissue culture induced variability in ginger was also reported (Suja, 2002; Nirmal Babu *et al.*, 2003). These authors reported clonal variability in the micropropagated ginger plants through molecular characterization.

4.3.1.3. Callus regenerated plants

RAPD profile of callus regenerated plants exhibited polymorphism with a number of primers tried. Primer OPB 7 showed polymorphism in the banding pattern of callus regenerated plants of the var. Varada. In the RAPD profile of the callus regenerated plants vs. aerial stem and vegetative bud regenerated plants, the absence of one band of size 2200 bp was noticed. However, there was no intra plant variability in the callus regenerated ginger plants with this primer (Fig. 7. g) (Table 24).

Primer OPB 10 produced one polymorphic band in the RAPD profile of callus regenerated samples of the var. Jamaica and var. Varada. One DNA fragment with 1250 and 1900 bp were absent in the one callus regenerated samples of var. Jamaica (Lane 11) and var. Varada (Lane 16) (Fig. 7. d & h). In the case of OPB 11, two DNA fragments with the size of 1900 bp and 1800 bp were absent in the RAPD profile of callus regenerated plants of the var. Varada (Fig. 8. d). Callus regenerated plants of the var. Varada showed good polymorphism in the RAPD profile of primer OPB 15. Two bands with the size of 1800 bp and 1400 bp were absent and two additional bands with the size of 500 bp and 600 bp were present in these samples (Fig. 8. e). These primers however, failed to produce polymorphism in the var. Jamaica (Fig.8. b) (Table 24).

RAPD profile of primer OPD 13 produced two polymorphic band in the callus regenerated samples of the var. Jamaica. Eight bands were monomorphic across all the samples but one band with the size of 2200 and 1400 bp was absent in this group (Fig. 9. d).

Callus regenerated plants of the var. Varada showed polymorphism in the RAPD profile of primer OPJ 1. Two amplicons with the size of 2000 bp and 600 bp were absent in the last four samples of callus regenerated plants (Lane 13 – 16). One amplicon (1200 bp) was not present in the first callus regenerated sample (Lane 12) (Fig. 10. d). Primer OPJ 7 produced good polymorphism in callus regenerated samples of the var. Varada. DNA fragments with 750 bp and 500 bp were absent in callus regenerated samples. Two bands with 1000 bp and 850 bp were unique to first sample of callus regenerated plants (Lane 12) (Fig. 10. e). These primers failed to produce polymorphism in the var. Jamaica (Fig. 10. b) (Table 24).

Primers OPB 1, OPB 6, OPB 9, OPB 14, OPB 17, OPD 2, OPD 3, OPD 11, OPJ 6, OPJ 10 and OPJ 16 were failed to produce polymorphism in the callus regenerated samples of both the varieties (Table 24).

The intraclonal polymorphism observed in the callus regenerated plants may be due to somaclonal variations occurred during *in vitro* culturing of plant tissues. Several mechanisms may be responsible for the induction of somaclonal variation. These include the gross karyotypic changes which accompany *in vitro* culture via calli, chromosomal rearrangements, somatic crossing over with sister chromatid exchange, gene amplification or diminution or perhaps various combinations of these processes (George, 1993a; Vasil, 1986).

The cell and callus cultures on periodical subculture undergo various morphological and genetic changes like, polyploidy, aneuploidy and mutations (Nagl, 1972; D' Amato, 1985). Further, both polyploidy and aneuploidy may arise as a result of the use of synthetic auxin like 2,4-D in culture medium, more than any other growth regulators (Singh and Harvey, 1975; D'Amato, 1978). These compounds are known to induce spindle failure and other abnormalities of mitosis in intact plants. The tissue culture environment may enhance the frequency of somatic crossing over, and if a proportion of such an exchange is asymmetric or between non homologous

chromosomes, then genetic variants could be generated as a consequence (Ignacimuthu, 1999).

In the present study, variations were observed with the callus regenerated plants only and the plants regenerated from aerial stem and vegetative buds were genetically uniform and true to type. This may be due to the vulnerability of callus (undifferentiated tissue) towards the *in vitro* climatic conditions and media components. According to Gould (1986), rate of cell division is very important in the accumulation of mutation. Rate of cell division is slower in direct regenerated plants compared to callus regenerated method. In direct regenerated plants, cells are organized and differentiation process is controlled more stringently than in callus cultures. These two factors along with the use of 2,4-D and cytokinins for callus induction and regeneration should explain the higher variation observed in callus regenerated plants.

Salvi *et al.* (2003) observed that plants regenerated directly from shoot tips of turmeric were more uniform but callus regenerated plants showed polymorphism in the RAPD profile. Genetic variability in the callus regenerated plants was also observed by RAPD analysis of turmeric (Salvi *et al.*, 2001; Praveen, 2005).

Random amplified polymorphic DNA (RAPD) analysis of *in vitro* regenerated turmeric plantlets revealed 103 scorable bands from 10 primers, including nine polymorphic bands, which were absent in control. However, most of the regenerated plantlets were similar to the mother plants (Prakash *et al.*, 2004). Salvi *et al.* (2001) reported that Random Amplified Polymorphic DNA (RAPD) analysis of eight regenerated plants using 14 primers when separated on non-denaturing polyacrylamide gels showed 38 novel bands. About 51 bands present in the control were absent in the regenerants. The result indicates that variation at DNA level has occurred during *in vitro* culture.

A number of RAPD primers failed to produce polymorphism in the callus regenerated plantlets. Hence the failure of RAPD analysis to detect intraclonal polymorphism in the experiment need not be taken as definite proof of the absence of such variation. RAPD markers are reported to fail to detect polymorphism associated with variation at the phenotypic, epigenetic or ploidy level (Isabel *et al.*, 1996; Fourre *et al.*, 1997; Hao *et al.*, 2004).

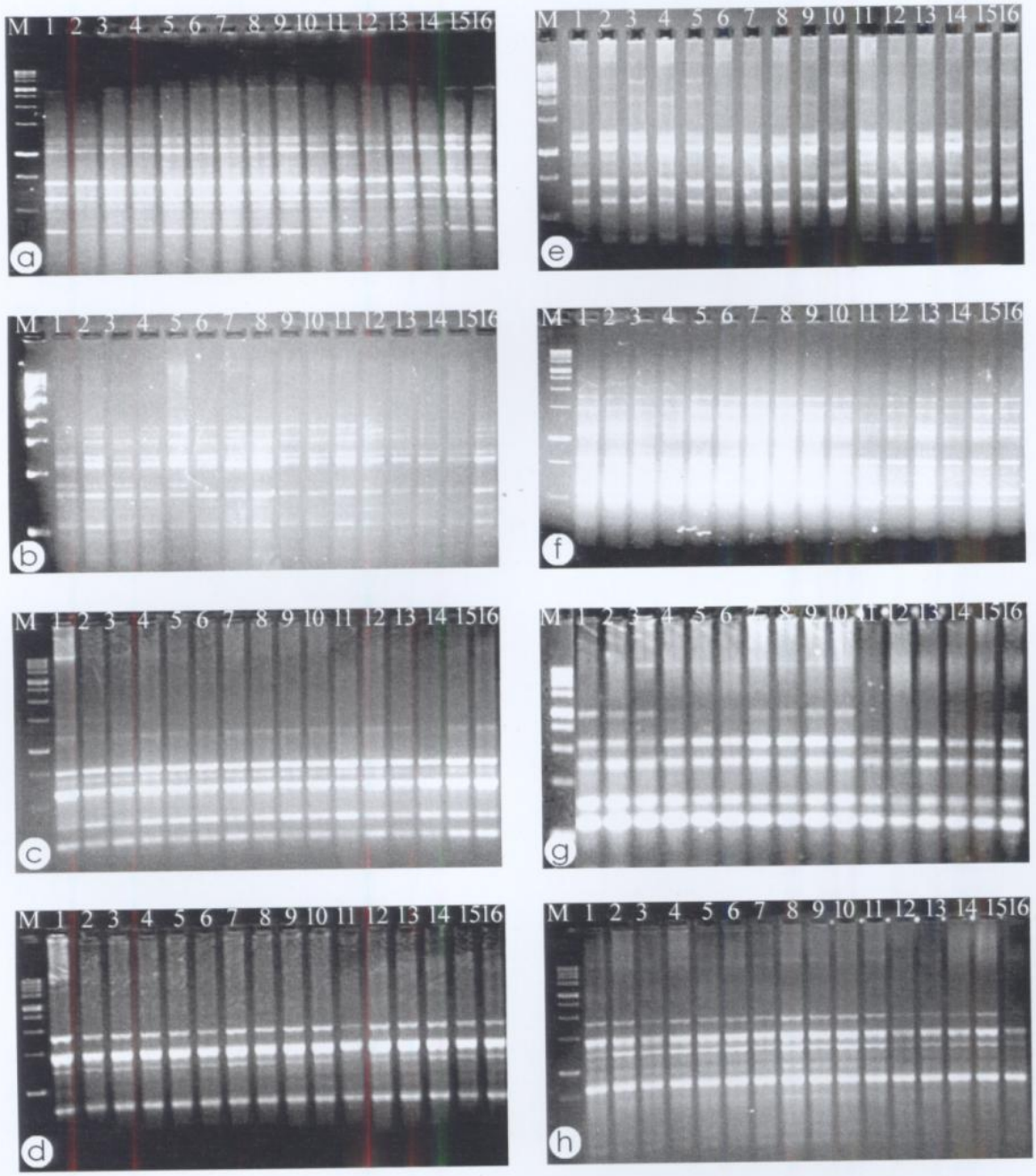


Fig. 7. a- d & e - f are RAPD profiles of *in vitro* derived plants of var. Jamaica and var. Varada using primers OPB 01, OPB 06, OPB 07 and OPB 10 respectively. M- marker (1 Kb ladder), 1- control, 2 to 6 - aerial stem regenerated plants, 7 to 11 - vegetative bud derived plants and 12 to 16 - callus regenerated plants.

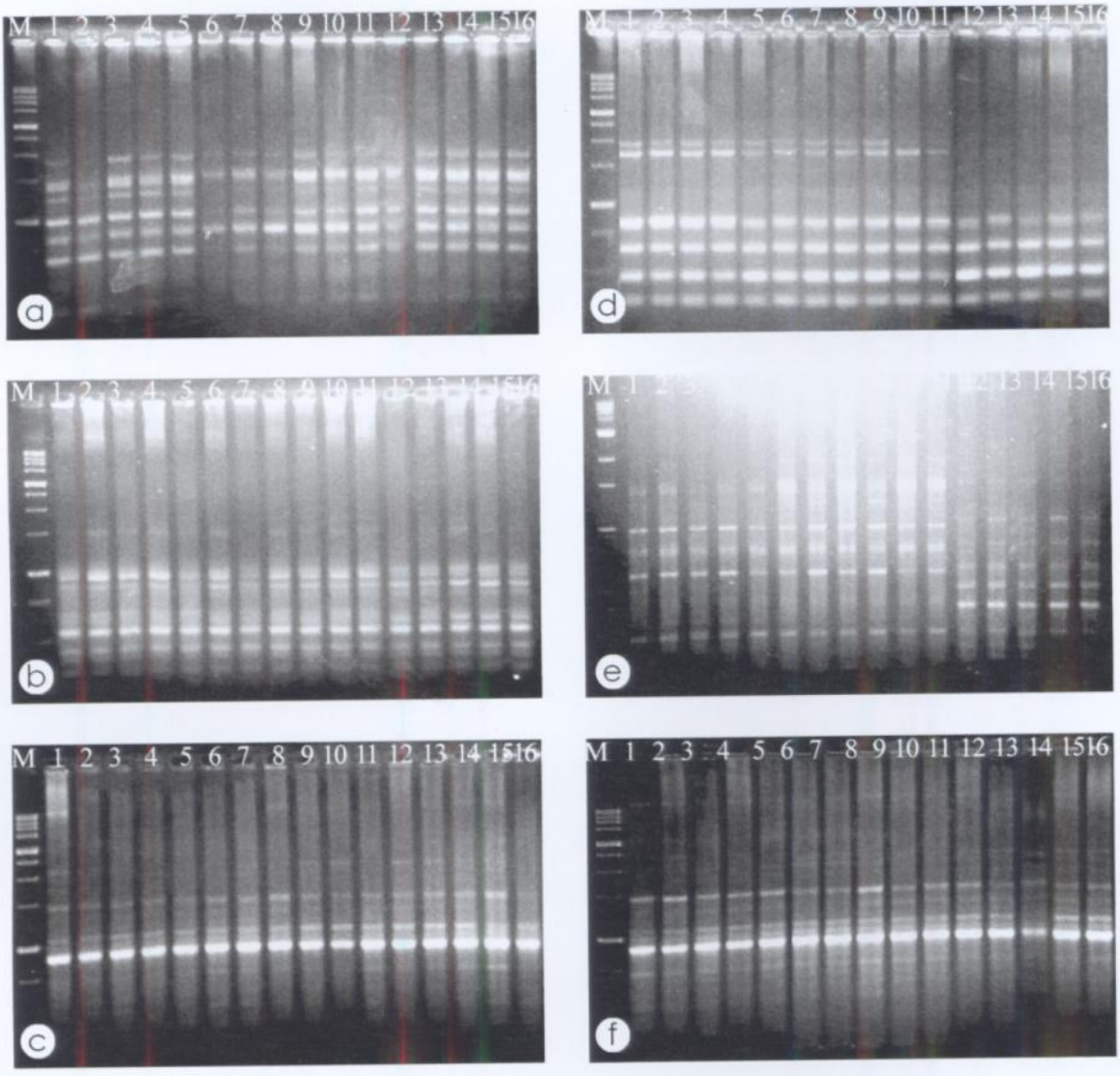


Fig. 8. a - c are RAPD profiles of *in vitro* derived plants of var. Jamaica using primers OPB 14, OPB 15 and OPB 17, respectively. d - f are RAPD profiles of *in vitro* derived plants of var. Varada using primers OPB 11, OPB 15 and OPB 17 respectively. M- marker (1 Kb ladder), 1- control, 2 to 6 - aerial stem regenerated plants, 7 to 11 - vegetative bud derived plants and 12 to 16 - callus regenerated plants.

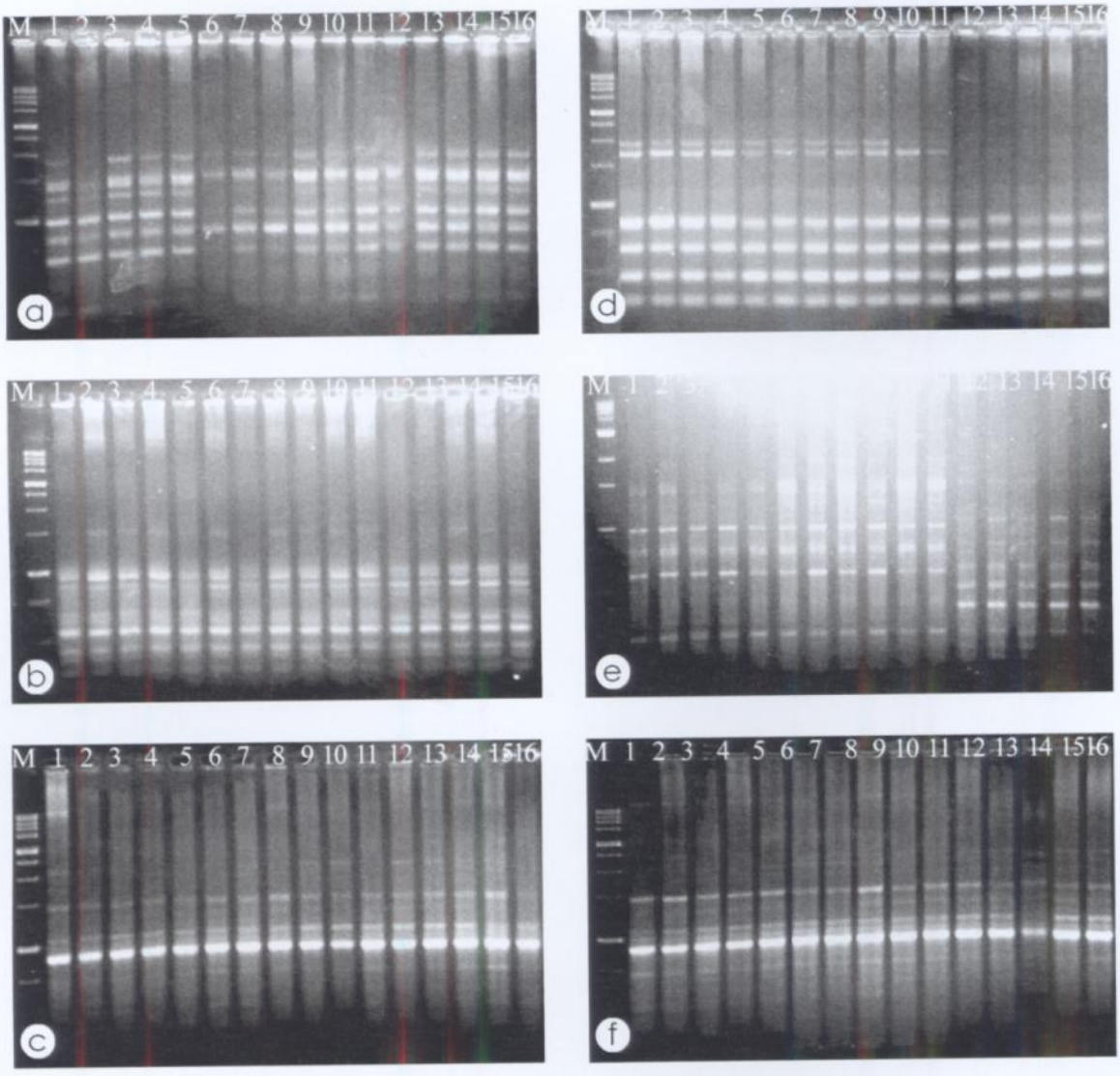


Fig. 8. a - c are RAPD profiles of *in vitro* derived plants of var. Jamaica using primers OPB 14, OPB 15 and OPB 17, respectively. d - f are RAPD profiles of *in vitro* derived plants of var. Varada using primers OPB 11, OPB 15 and OPB 17 respectively. M- marker (1 Kb ladder), 1- control, 2 to 6 - aerial stem regenerated plants, 7 to 11 - vegetative bud derived plants and 12 to 16 - callus regenerated plants.

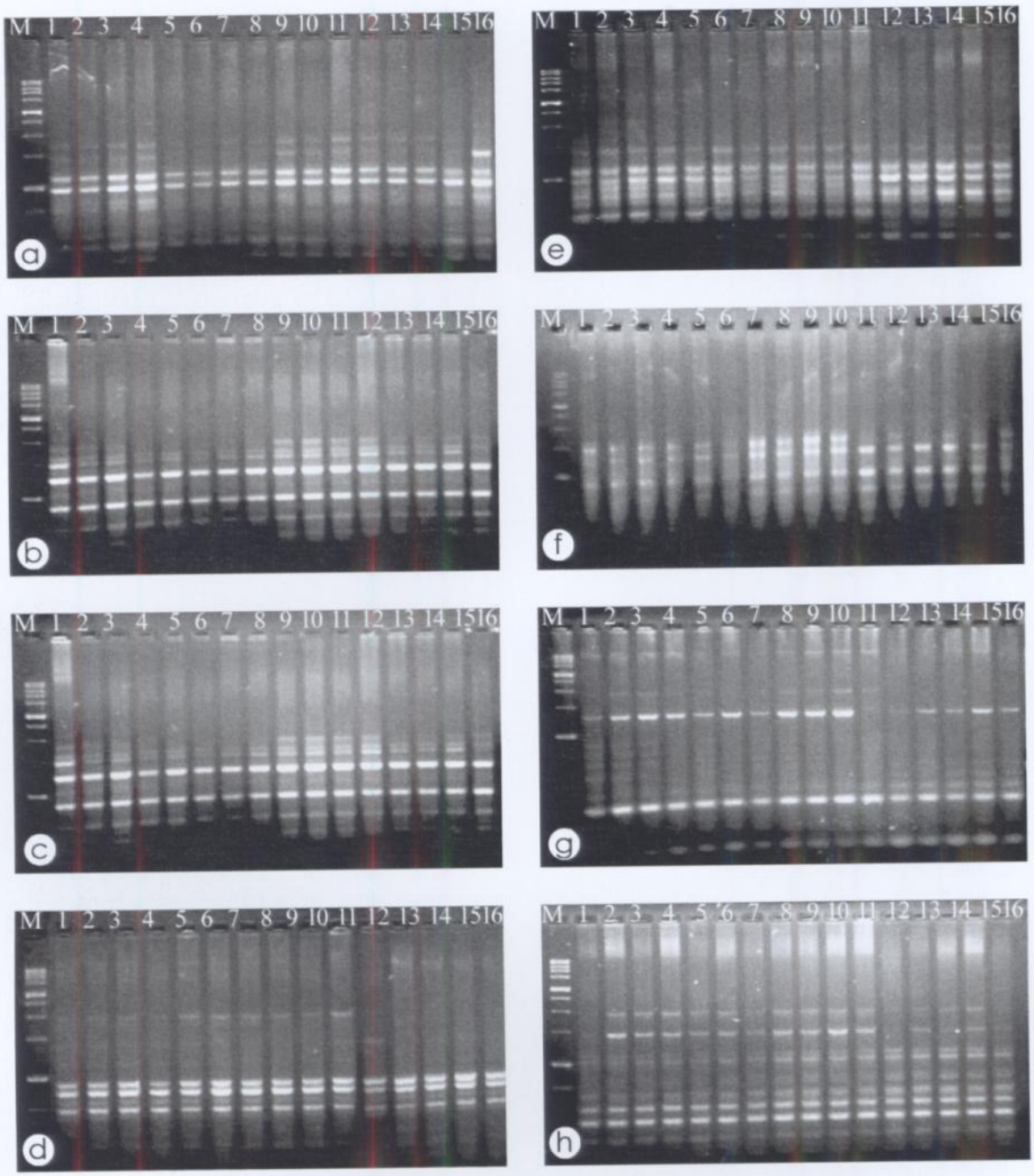


Fig. 9. a- d and e-h are RAPD profiles of *in vitro* derived plants of var. Jamaica and var. Varada using primers OPD 02, OPD 03, OPD 11 and OPD 13, respectively. M- marker (1 Kb ladder), 1- control, 2 to 6 - aerial stem regenerated plants, 7 to 11 - vegetative bud derived plants and 12 to 16 - callus regenerated plants.

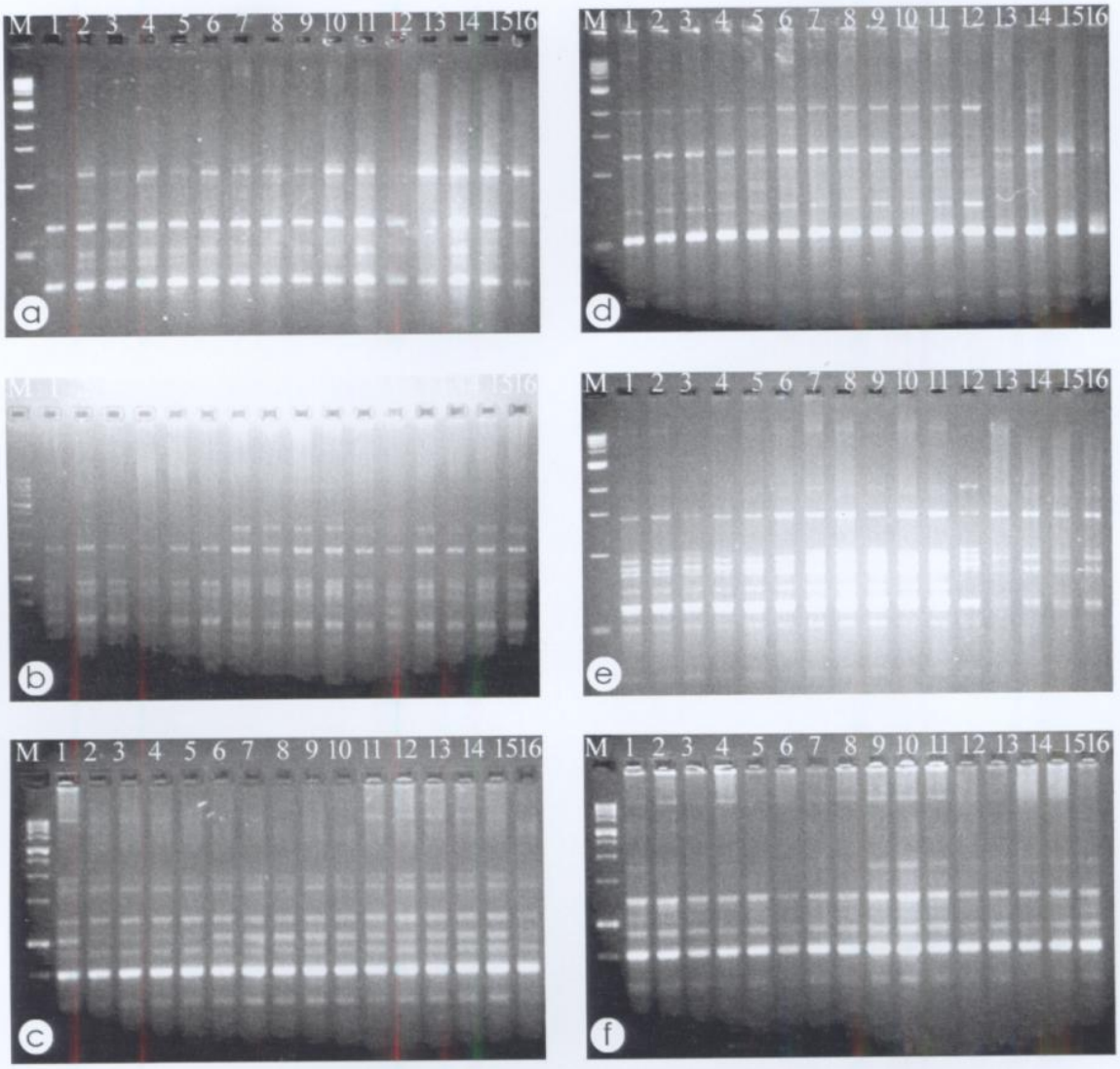


Fig. 10. a- c and d-f are RAPD profiles of *in vitro* derived plants of var. Jamaica and var. Varada using primers OPJ 01, OPJ 07 and OPJ 10, respectively. M- marker (1 Kb ladder), 1- control, 2 to 6 - aerial stem regenerated plants, 7 to 11 - vegetative bud derived plants and 12 to 16 - callus regenerated plants.

4.4. Hardening

The micropropagated plants were acclimatized at room temperature (Fig. 11. a).

4.4.1. Plant survival

In the case of plant survival, all treatments except treatment 2 (coir dust + polythene covering) gave good result (90 – 100%). Plants grown in this treatment (Treatment 2) resulted in only 70 % survival. However, Samsudeen (1996) reported that survival of plants covered with poly bags was better than that were not covered. Micropropagated plants hardened in treatment containing *Trichoderma* showed 100% survival as compared to others. Earlier reports also suggested that the use of antagonistic biological agents in the hardening medium resulted in better establishment, survival and growth of micropropagated plants after transfer to *ex vitro* conditions (Pandey, 2002).

4.4.2. Plant height

Analysis of variance for plant height revealed the significant effects of variety and treatment for height of the plants. Effect of variety X treatment interaction was found non significant (Table 25).

Table 25. Analysis of variance for plant height and number of leaves.

Source	Degrees of freedom	Plant height			No. of leaves		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Replication	4	9.5	2.4	0.54^{ns}	1.7	0.4	0.17^{ns}
Variety	1	47.4	47.4	10.8**	0.0	0.0	0.00^{ns}
Treatment	4	1053.2	263.3	59.7**	241.3	60.3	23.64**
Variety X Treatment	4	46.1	11.5	2.62^{ns}	40.6	10.2	3.98**
Error	36	158.8	4.4		91.9	2.6	
Total	49	1315.2			375.5		

Mean plant height of the hardened plants grown in different medium are presented in Table 26 and the mean values for varieties showed that the var. Jamaica had longer shoots than the var. Varada. Among the five treatments tried, treatment 5 (Soil: Sand: coir dust: Cow dung + 5g *Trichoderma* / Cup) gave good result followed by treatment 4 (soil: sand: coir dust: cow dung) in both the varieties studied (Table 26) (Fig. 11. b). Plant height was minimum in the plants grown in the treatment 2 (Sterilized coir dust + polythene covering).

Table 26. Mean plant height, no. of leaves and chlorophyll content of the plantlets hardened in different medium.

Treatments*	Plant height (cm)			No. of leaves			Chlorophyll[a] (mg)			Chlorophyll[b] (mg)			Total chlorophyll (mg)		
	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean
1	10.8	9.30	10.1	8.00	8.40	8.2	0.49	0.65	0.57	0.28	0.30	0.29	0.78	0.95	0.87
2	6.40	8.00	7.2	7.40	9.20	8.3	0.56	0.59	0.58	0.22	0.22	0.22	0.77	0.81	0.79
3	15.0	11.5	13.3	11.2	9.00	10.1	0.71	0.70	0.71	0.26	0.30	0.28	0.97	1.00	0.99
4	15.7	12.9	14.3	12.0	10.0	11	1.00	0.81	0.91	0.41	0.36	0.39	1.40	1.16	1.28
5	22.6	19.0	20.8	13.2	15.2	14.2	1.22	1.19	1.21	0.51	0.48	0.5	1.73	1.67	1.70
Mean	14.1	12.1		10.4	10.4		0.8	0.79		0.34	0.33		1.13	1.12	
CD (P=0.05)															
Variety	0.86			NS			NS			NS			NS		
Treatment	1.36			1.03			0.04			0.02			0.05		
Variety X Treatment	NS			1.46			0.06			0.29			0.07		
CV (%)	16.00			15.42			7.50			6.72			6.75		

*Treatments: 1) sterilized coir dust, 2) sterilized coir dust + polythene covering, 3) sterilized coir dust + Trichoderma @ 5 g/cup (CFU = 10^{14} g^{-1}), 4) soil: sand: coir dust: cow dung (sterilized), 5) soil: sand: coir dust: cow dung (sterilized): Trichoderma @ 5 g/cup.

4.4.3. Number of leaves

Effects of treatment and variety X treatment interaction for number of leaves were found highly significant. But the effect of variety was not significant (Table 25).

Mean number of leaves in the hardened plants grown in different medium are given in the Table 26. Maximum number of leaves were observed in the plants grown in treatment 5 (soil: sand: coir dust: cow dung + 5g *Trichoderma* / Cup) followed by treatment 4 (soil: sand: coir dust: cow dung). Minimum number of leaves was observed in plants grown in treatment 2 (coir dust + polythene covering) in case of the var. Jamaica and in treatment 1 (coir dust) in case of the var. Varada (Table 26).

Earlier studies indicated that inoculation of plantlets with *Piriformospora indica* during acclimatization reduced the stress of acclimatization, providing faster growth and better establishment (more than 90 %) of micropropagated plants of *Nicotiana tobacum* L. and *Bacopa monniera* L. (Singh *et al.*, 2000).

Treatments with endomycorhiza (*Glomus etunicatum*) at the *ex vitro* transferring stage was beneficial for acclimatization, improving plant growth and development of *Curcuma zedoaria* Rosc. (Miachir *et al.*, 2004).

4.4.4. Chlorophyll content – chlorophyll [a]

The effects of treatment and variety X treatment interaction were found significant for chlorophyll [a] content but the effect of variety was found non significant (Table 27).

Table 27. Analysis of variance for chlorophyll[a] , chlorophyll[b] and total chlorophyll content.

Source	Degrees of freedom	Chlorophyll[a]			Chlorophyll[b]			Total chlorophyll		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Replication	4	0.0	0.00	0.03 [ns]	0.00	0.00	2.23*	0.00	0.00	0.12 [ns]
Variety	1	0.0	0.00	0.39 [ns]	0.00	0.00	0.83 [ns]	0.00	0.00	0.57 [ns]
Treatment	4	2.8	0.71	201.49**	0.48	0.12	236.75**	5.56	1.39	240.84**
Variety X Treatment	4	0.16	0.04	11.06**	0.01	0.00	5.65**	0.23	0.06	9.84**
Error	36	0.13	0.00		0.02	0.00		0.21	0.01	
Total	49	3.12			0.51			6.0		

Mean values of chlorophyll [a] content in the hardened plants grown in different medium are given in the Table 26. It was observed that, chlorophyll [a] was more in the plants grown in treatment 5 (soil: sand: coir dust: cow dung + 5g *Trichoderma* / Cup) followed by treatment 4 (soil: sand: coir dust: cow dung) for both the varieties. Minimum chlorophyll [a] content was observed in plants grown in treatment 1 (coir dust) in the var. Jamaica and in treatment 2 (coir dust + polythene covering) in the var. Varada (Table 26).

4.4.5. Chlorophyll [b] content

Effects of treatment and variety X treatment interaction were found significant for chlorophyll [b] content in the hardened plants. The effect of varieties was non significant here also (Table 27).

Mean values of chlorophyll [b] content in the hardened plants grown in different medium are given in the Table 26. The maximum chlorophyll [b] content was observed in the plants grown in treatment 5 (soil: sand: coir dust: cow dung + 5g *Trichoderma* / Cup) followed by treatment 4 (soil: sand: coir dust: cow dung) and the minimum was in treatment 2 (coir dust + polythene covering) for both the varieties (Table 26).

4.4.6. Total chlorophyll

Total chlorophyll content varied significantly with the different treatments tried. The variety X treatment interaction was found highly significant but the effect of varieties was not significant for total chlorophyll content as in the case of chlorophyll[a] chlorophyll[b] compounds (Table 27).

Mean values of total chlorophyll content in the hardened plants grown in different medium are given in the Table 26. The maximum total chlorophyll was found in plants grown in treatment 5 (Soil: Sand: coir dust: Cow dung + 5g *Trichoderma* / Cup) followed by treatment 4 (Soil: sand: coir dust: cow dung) and the

minimum was in treatment 2 (coir dust + polythene covering) in both varieties studied (Table 26).

The use of *Trichoderma* along with an organic fertilizer for the plantlet hardening might be responsible for the good performance observed in case of the plants grown in the treatment 5 (soil: sand: coir dust: cow dung + 5g *Trichoderma* / Cup).

Krishna *et al.* (2005) reported that the mycorrhizal inoculation at an early stage resulted in the accumulation of different biochemicals in the plant system such as chlorophyll, carotenoids, proline, phenol and enzymes like polyphenol oxidase and nitrate reductase. These plantlets showed enhanced survival and improved tolerance against stresses experienced during weaning phase. The mycorrhizal plants also exhibited improved physiological and nutritional status and had higher relative water content and photosynthetic rate.

4.5. Field evaluation

The hardened plantlets were transferred to polythene bags (30 x 40 cm) under protected condition, in an experimental shed with 50% shade (Fig. 11. c & d).

4.5.1. Morphological and yield characteristics of in vitro *derived plants*

The morphological and yield characteristics of the plants for the three years study (2003-2006) were analyzed statistically and the results are given below:

4.5.1.1. Plant height

Analysis of variance for plant height revealed significant effects of varieties, explants and variety X explant interaction for plant height (Table 28).

Table 28. Analysis of variance for plant height in field grown tissue cultured plants.

Source	Degrees of freedom	2003-2004			2004-2005			2005-2006		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Variety	1	798.85	798.85	45.12**	497.01	497.01	25.85**	305.37	305.37	16.32**
Explant	3	1281.9	427.31	24.14**	4652.20	1550.73	80.68**	735.52	586.58	31.34**
Variety X Explant	3	567.19	189.06	10.68**	671.38	223.79	11.64**	1759.74	245.17	13.10**
Error	72	1274.73	17.71		1383.91	19.22		1347.54	18.72	
Total	79	3922.68			7204.45			4148.17		

Mean values of plant height recorded from the field grown ginger plants, during three years of study are given in the Tables 29, 30 and 31. The results indicated that the var. Varada produced taller tillers compared to the var. Jamaica in all the three years of study (Tables 29, 30 and 31).

Table 29. Mean values of morphological characters recorded from the field grown ginger plants during the year 2003 – 2004.

Treatments	Plant height (cm)			No. of tiller			No. of leaves			Leaf length (cm)			Leaf width (cm)		
	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean
1	63.0	69.2	66.1	11.2	13.1	12.2	20.6	20.8	20.7	20.7	21.4	21.1	2.6	2.7	2.7
2	60.9	62.7	61.8	10.9	10.9	10.9	19.5	19.5	19.5	20.8	20.5	20.7	2.6	2.6	2.6
3	56.0	59.6	57.8	7.6	7.2	7.4	15.1	15.6	15.35	19.5	17.9	18.7	2.5	2.3	2.4
4	62.1	75.9	69.0	6.7	7.5	7.1	16.5	18.3	17.4	19.4	23.4	21.4	2.7	2.8	2.7
Mean	60.5	66.9		9.1	9.7		17.9	18.6		20.1	20.8		2.6	2.6	
CD (P=0.05)															
Variety	1.89			NS			NS			0.45			NS		
Explant	2.67			1.38			1.18			0.63			0.10		
Variety X Explant	3.77			NS			NS			0.89			NS		
CV (%)	6.61			23.1			10.2			4.88			5.93		

Treatments: 1) Aerial stem regenerated plants, 2) Vegetative bud regenerated plants, 3) Callus regenerated plants, 4) Control plants.

Table 30. Mean values of morphological characters recorded from the field grown ginger plants during the year 2004-2005.

Treatment	Plant height (cm)			No. of tiller			No. of leaves			Leaf length (cm)			Leaf width (cm)		
	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean
1	71.9	73.9	72.9	13.8	12.4	13.1	20.1	18.7	19.4	20.9	21.6	21.3	2.6	2.7	2.7
2	64.6	62.3	63.5	9.4	9.1	9.3	17.8	17.1	17.5	20.7	19.8	20.3	2.6	2.4	2.5
3	50.0	54.9	52.5	8.3	8.4	8.4	15.7	16.9	16.3	18.6	19.4	19.0	2.2	2.2	2.2
4	57.3	70.6	64.0	6.8	6.3	6.6	17.6	18.3	18.0	18.3	23.6	21.0	2.8	2.9	2.9
Mean	61.0	65.4		9.6	9.1		17.8	17.8		19.6	21.1		2.6	2.6	
CD (P=0.05)															
Variety	1.96			NS			NS			0.55			NS		
Explant	2.78			1.58			1.67			0.78			0.14		
Variety X Explant	3.93			NS			NS			1.10			0.20		
CV (%)	6.96			26.8			14.8			6.02			8.69		

Treatments: 1) Aerial stem regenerated plants, 2) Vegetative bud regenerated plants, 3) Callus regenerated plants, 4) Control plants.

Table 31. Mean values of morphological characters recorded from the field grown ginger plants during the year 2005 – 2006.

Treatment	Plant height (cm)			No. of tiller			No. of leaves			Leaf length (cm)			Leaf width (cm)		
	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean
1	72.8	75.6	74.2	13.9	11.6	12.8	21.4	20.9	21.2	21.6	21.7	21.7	2.7	2.74	2.7
2	68.3	68.6	68.5	8.7	8.2	8.5	19.6	19.8	19.7	21.8	20.7	21.35	2.6	2.69	2.7
3	61.9	60.4	61.2	8.5	8.3	8.4	16.3	17.8	17.1	19.8	18.2	19.0	2.5	2.50	2.5
4	62.8	76.9	69.9	8.2	8.5	8.4	19.7	20.7	20.2	19.7	23.6	21.7	2.8	2.87	2.8
Mean	66.5	70.4		9.8	9.2		19.3	19.8		20.7	21.1		2.7	2.7	
CD (P=0.05)															
Variety	1.94			NS			NS			NS			0.06		
Explant	2.74			1.29			1.15			0.59			0.09		
Variety X Explant	3.88			NS			NS			0.84			NS		
CV (%)	6.32			21.4			9.26			4.49			5.14		

Treatments: 1) Aerial stem regenerated plants, 2) Vegetative bud regenerated plants, 3) Callus regenerated plants, 4) Control plants.

Among the different explant sources, the aerial stem derived plants exhibited maximum plant height in the var. Jamaica in all the three years followed by the control plants (conventionally propagated) during the year 2003-2004 and the vegetative bud derived plants in the years 2004-05 and 2005-06. Minimum plant height was observed in the callus regenerated plants in all the three years (Tables 29, 30 and 31).

However, in the var. Varada, maximum plant height was observed in the conventionally propagated plants followed by the aerial stem regenerated plants in the years 2003-04 and 2005-06. However, in the year 2004-05, the aerial stem derived plants showed maximum plant height followed by control plants. Minimum plant height was observed in the callus regenerated plants (Tables 29, 30 and 31).

4.5.1.2. Number of tillers

Total number of tillers varied significantly with the type of explant used for plant regeneration. The effects of varieties and variety X explant interaction were found non significant (Table 32).

Table 32. Analysis of variance for tiller number in field grown tissue cultured plants.

Source	Degrees of freedom	2003-2004			2004-2005			2005-2006		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Variety	1	6.62	6.61	1.45^{ns}	5.51	5.51	0.88^{ns}	9.11	9.11	2.20^{ns}
Explant	3	382.04	127.35	27.06**	458.14	152.71	24.46**	283.94	94.65	22.89**
Variety X Explant	3	15.44	5.15	1.09^{ns}	6.04	2.01	0.32^{ns}	19.24	6.41	1.55^{ns}
Error	72	338.90	4.71		449.50	6.24		297.70	4.14	
Total	79	742.99			919.19			609.99		

Mean values of tiller number recorded from the field grown ginger plants, during three years of study are given in the Tables 29, 30 and 31. Mean values for varieties showed that the var. Varada produced more tillers during the year 2003-

2004 and the var. Jamaica produced more number of tillers compared to the var. Varada during the years 2004-2005 and 2005-2006 (Tables 29, 30 and 31).

In the var. Jamaica, maximum number of tillers was observed in the plants regenerated from aerial stem followed by vegetative buds and the callus regenerated plants. Minimum tiller number was observed in the control plants (Tables 29, 30 and 31).

In the var. Varada, the aerial stem regenerated plants registered maximum tiller number in all the years of study. However, second highest tiller number was recorded from the vegetative bud derived plants (2003-04; 2004-05) and in the control plants (2005-06). Minimum number of tillers was observed in the callus regenerated plants during the year 2003-04, the control plants in the year 2004-05 and the vegetative bud derived plants in the year 2005-06 (Tables 29, 30 and 31).

4.5.1.3. Number of leaves

Number of leaves varied significantly with the type of explants used for plant regeneration but the effects of varieties and variety X explant interaction were not significant (Table 33).

Table 33. Analysis of variance for number of leaves in field grown tissue cultured plants.

Source	Degrees of freedom	2003-2004			2004-2005			2005-2006		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Variety	1	7.81	7.81	2.26^{ns}	0.05	0.05	0.007^{ns}	6.05	6.05	1.85^{ns}
Explant	3	333.94	111.31	32.20**	99.05	33.02	4.78**	183.25	61.08	18.68**
Variety X Explant	3	9.84	3.28	0.95^{ns}	21.85	7.28	1.06^{ns}	13.25	4.42	1.35^{ns}
Error	72	248.90	3.46		497.00	6.90		235.40	3.27	
Total	79	600.49			617.95			437.95		

The mean values of leaf number recorded from the field grown ginger plants, during three years of study are given in the Tables 29, 30 and 31. The results showed that the var. Varada produced more number of leaves during the years 2003-2004 and 2005-2006 (Tables 29 and 31). However, in the year 2004-2005, both the varieties produced equal number of leaves (Table 30).

Among the four explants tried, maximum number of leaves were observed in the plants regenerated from aerial stem followed by vegetative buds and minimum in the callus regenerated plants in all the three years of study (var. Jamaica). However, in the var. Varada, the aerial stem derived plants produced maximum number of leaves followed by control plants (2004-05 and 2005-06) and vegetative bud regenerated plants (2003-04). Callus regenerated plants recorded minimum number of leaves in this case also (Tables 29, 30 and 31).

4.5.1.4. Leaf length

Analysis of variance for leaf length revealed significant effect of varieties, explant and variety X explant interaction for leaf length during the years 2003-04 and 2004-05. However, in the year 2005-06, the varietal effect was found non significant (Table 34).

Table 34. Analysis of variance for leaf length in field grown tissue cultured plants.

Source	Degrees of freedom	2003-2004			2004-2005			2005-2006		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Variety	1	10.44	10.44	10.51**	41.54	41.54	27.61**	2.49	2.49	2.82^{ns}
Explant	3	83.75	27.92	28.10**	61.74	20.58	13.68**	97.96	32.65	37.09**
Variety X Explant	3	84.24	28.08	28.26**	105.80	35.27	23.44**	95.00	31.67	35.98**
Error	72	71.53	0.99		108.32	1.50		63.38	0.88	
Total	79	249.96			317.40			258.83		

Mean values of leaf length recorded from the field grown ginger plants, during three years of study are given in the Tables 29, 30 and 31. Among the two

varieties studied, the var. Varada showed maximum leaf length as compared to the var. Jamaica in all the three years of study.

In the var. Jamaica, maximum leaf length was observed in the plants regenerated from vegetative buds followed by the aerial stem regenerated plants in the years 2003-04 and 2005-06. During the year 2004-05, the aerial stem regenerated plants showed maximum leaf length. Minimum leaf length was observed in the callus regenerated plants in all the three years (Tables 29, 30 and 31).

However, in the var. Varada, maximum leaf length was observed in the control plants followed by the aerial stem regenerated plants and minimum leaf length was observed in the callus regenerated plants in all the three years of study (Table 29, 30 and 31).

4.5.1.5. Leaf width

Width of leaves was influenced significantly by the type of explants used for plant regeneration in all the three years but the varietal effect was significant only in the year 2005-06. The interaction between variety and explant was significant only in the year 04-05 (Table 35).

Table 35. Analysis of variance for leaf width in field grown tissue cultured plants.

Source	Degrees of freedom	2003-2004			2004-2005			2005-2006		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Variety	1	0.004	0.004	0.16^{ns}	0.04	0.04	0.87^{ns}	0.10	0.10	5.42*
Explant	3	0.695	0.232	9.66**	4.82	1.61	32.55**	1.16	0.39	20.56**
Variety X Explant	3	0.128	0.043	1.78^{ns}	0.55	0.18	3.70*	0.05	0.02	0.82^{ns}
Error	72	1.727	0.024		3.55	0.05		1.35	0.02	
Total	79	2.553			8.96			2.65		

The mean values of leaf width recorded from the field grown ginger plants, during three years of study are given in the Tables 29, 30 and 31. For this trait, both the varieties exhibited almost same leaf width in all the three years (Tables 29, 30 and 31).

In both the varieties, maximum leaf width was observed in the control plants followed by the plants regenerated from aerial stem. The callus regenerated plants showed minimum leaf width in all the three years of study as observed in case of leaf length (Tables 29, 30 and 31).

4.5.1.6. Number of cormlets

Number of cormlets in a whole rhizome varied significantly with the explants used for plant regeneration. The effect of varieties was non significant for all the three years and variety X explant interaction was significant only in the year 2005-06 (Table 36).

Table 36. Analysis of variance for number of cormlets in field grown tissue cultured plants.

Source	Degrees of freedom	2003-2004			2004-2005			2005-2006		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Variety	1	1.513	1.51	0.26^{ns}	4.51	4.51	0.89^{ns}	6.61	6.61	1.35^{ns}
Explant	3	1225.94	408.65	70.71**	1240.12	413.38	82.02**	1741.94	580.65	118.67**
Variety X Explant	3	35.14	11.71	2.03^{ns}	21.84	7.28	1.44^{ns}	66.54	22.18	4.53**
Error	72	416.10	5.78		362.90	5.04		352.30	4.89	
Total	79	1678.67			1629.39			2167.39		

The mean values of number of cormlets recorded from the field grown ginger plants, during three years of study are given in the Tables 37,38 and 39. For this trait, both the varieties showed approximately same number of cormlets in all the three years.

During the year 2003-2004, in the var. Jamaica, the aerial stem regenerated plants registered maximum number of cormlets followed by the control plants. However, the trend was reversed during the years 2004-2005 and 2005-2006, in the var. Jamaica and in all the three years of study in the var. Varada. The callus regenerated plants produced minimum number of cormlets, in both the varieties (Tables 37, 38 and 39).

Table 37. Mean values of yield attributes recorded from the field grown ginger plants during the year 2003 – 2004.

Treatment	No. of cormlets			Length of cormlets (cm)			Circumference of cormlets (cm)			No. of nodes/cormlet			Length of inter nodes (cm)			Weight of rhizomes (g)		
	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean
1	14.7	13.1	13.9	3.3	3.3	3.3	6.5	4.4	5.45	5.4	5.6	5.5	0.5	0.6	0.55	52.70	40.70	46.7
2	10.2	10.2	10.2	2.6	2.2	2.4	5.2	3.9	4.55	5.6	5.2	5.4	0.5	0.5	0.5	46.70	38.90	42.8
3	3.80	5.9	4.85	2.0	2.4	2.2	3.2	3.7	3.45	4.3	4.9	4.6	0.4	0.9	0.65	16.15	12.90	14.5
4	14.5	15.1	14.8	3.8	4.1	3.95	7.1	7.2	7.15	7.0	5.8	6.4	0.6	0.7	0.65	73.70	88.50	81.1
Mean	10.8	11.1		2.9	3.0		5.5	4.8		5.6	5.4		0.50	0.68		47.3	45.3	
CD (P=0.05)																		
Variety	NS			NS			0.21			NS			NS			NS		
Explant	1.52			1.05			0.30			0.42			NS			4.75		
Variety X Explant	NS			NS			0.42			0.59			NS			6.72		
CV (%)	21.9			55.6			9.12			12.0			87.5			16.2		

Treatments: 1) Aerial stem regenerated plants, 2) Vegetative bud regenerated plants, 3) Callus regenerated plants, 4) Control plants.

Table 38. Mean values of yield attributes recorded from the field grown ginger plants during the year 2004 – 2005.

Treatment	No. of cormlets			Length of cormlets (cm)			Circumference of cormlets (cm)			No. of nodes/cormlet			Length of inter nodes (cm)			Weight of rhizomes (g)		
	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean
1	14.2	14.1	14.15	3.8	2.4	3.1	6.7	3.86	5.28	7.6	5.23	6.415	0.58	0.49	0.54	54.30	36.80	45.55
2	11.4	10.7	11.05	3.0	2.2	2.6	5.2	4.02	4.61	6.9	5.16	6.03	0.59	0.45	0.52	43.10	35.56	39.33
3	3.8	5.9	4.85	1.9	1.9	1.9	3.2	3.74	3.47	4.3	4.92	4.61	0.44	0.48	0.46	16.15	12.90	14.53
4	14.5	15.1	14.8	3.9	4.1	4.0	7.1	7.23	7.17	7.0	5.79	6.40	0.59	0.65	0.62	73.70	88.50	81.10
Mean	11.0	11.5		3.15	2.65		5.55	4.71		6.45	5.28		0.55	0.52		46.8	43.4	
CD (P=0.05)																		
Variety	NS			0.25			0.34			0.31			NS			2.87		
Explant	1.42			0.35			0.47			0.44			0.06			4.05		
Variety X Explant	NS			0.50			0.67			0.62			0.08			5.73		
CV (%)	20.0			19.2			14.6			11.8			17.0			14.1		

Treatments: 1) Aerial stem regenerated plants, 2) Vegetative bud regenerated plants, 3) Callus regenerated plants, 4) Control plants.

Table 39. Mean values of yield attributes recorded from the field grown ginger plants during the year 2005 – 2006.

Treatment	No. of cormlets			Length of cormlets (cm)			Circumference of cormlets (cm)			No. of nodes/cormlet			Length of inter nodes (cm)			Weight of rhizomes (g)		
	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean
1	15.9	14.6	15.25	3.5	2.4	2.95	6.5	4.4	5.45	5.6	5.46	5.53	0.51	0.43	0.47	57.20	44.40	50.8
2	11.0	10.4	10.7	2.8	2.3	2.55	5.5	4.1	4.8	5.8	5.20	5.5	0.53	0.43	0.48	44.30	36.90	40.6
3	4.2	6.5	5.35	2.1	2.0	2.05	3.3	3.9	3.6	4.3	4.79	4.55	0.47	0.50	0.49	16.05	13.00	14.5
4	18.9	16.2	17.55	4.3	4.3	4.3	6.8	8.1	7.45	5.9	5.95	5.93	0.50	0.61	0.56	84.10	96.80	90.5
Mean	12.5	11.9		3.18	2.75		5.53	5.13		5.4	5.35		0.50	0.49		50.4	47.8	
CD(P=0.05)																		
Variety	NS			0.21			0.21			NS			NS			NS		
Explant	1.40			0.29			0.30			0.39			NS			5.59		
Variety X Explant	1.98			0.42			0.41			0.55			0.10			7.91		
CV (%)	18.1			15.9			8.77			11.5			22.2			18.0		

Treatments: 1) Aerial stem regenerated plants, 2) Vegetative bud regenerated plants, 3) Callus regenerated plants, 4) Control plants.

4.5.1.7. Length of cormlets

The analysis of variance for the length of cormlets in the year 2003-04 revealed significant influence of explants for the cormlet length. The varietal influence and variety X explant interaction were found non significant in this season. But in the following years (2004-2005 & 2005-2006), significant effects of varieties, explants and variety X explant interaction were observed for this attribute (Table 40).

Table 40. Analysis of variance for length of cormlets in field grown tissue cultured plants.

Source	Degrees of freedom	2003-2004			2004-2005			2005-2006		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Variety	1	0.14	0.14	0.05 ns	5.46	5.46	17.53**	3.34	3.34	14.95**
Explant	3	27.25	9.08	3.34*	44.53	14.84	47.65**	56.33	18.78	84.09**
Variety X Explant	3	14.03	4.68	1.72 ns	9.06	3.02	9.70**	4.01	1.34	5.98**
Error	72	195.95	2.72		22.43	0.31		16.08	0.22	
Total	79	237.38			81.49			79.75		

The mean values of cormlet length recorded from the field grown ginger plants, during three years of study are given in the Tables 37, 38 and 39. The results showed that the var. Varada showed highest length of cormlets during the year 2003-2004 and the var. Jamaica produced maximum cormlet length as compared to the var. Varada during the subsequent years (2004-2005 and 2005-2006).

In both the varieties, maximum cormlet length was observed in the control plants followed by the aerial stem regenerated plants and minimum cormlet length was recorded in the callus regenerated plants in all the three years of study (Tables 37, 38 and 39).

4.5.1.8. Circumference of cormlets

Circumference of cormlets varied significantly with varieties and explants used for plantlet regeneration. The interaction between varieties X explants was also found significant in all the three years of study (Table 41).

Table 41. Analysis of variance for circumference of cormlets in field grown tissue cultured plants.

Source	Degrees of freedom	2003-2004			2004-2005			2005-2006		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Variety	1	8.07	8.07	36.45**	13.25	13.25	23.68**	2.92	2.92	13.47**
Explant	3	145.45	48.49	219.19**	144.24	48.08	85.93**	152.78	50.93	234.74**
Variety X Explant	3	22.42	7.47	33.77**	36.32	12.11	21.64**	39.75	13.25	61.08**
Error	72	15.93	0.22		40.29	0.56		15.62	0.22	
Total	79	191.88			234.09			211.08		

The mean values of circumference of cormlets recorded from the field grown ginger plants, during three years of study are given in the Tables 37, 38 and 39. The results indicated that the var. Jamaica showed maximum circumference of cormlets compared to the var. Varada in all the three years of study.

Maximum circumference of cormlets was recorded in the control plants followed by the aerial stem derived plants and minimum was in callus regenerated plants in all the three years of study (var. Jamaica) and in the years 2003-04, 2005-06 (var. Varada). In the year 2004-05, the vegetative bud derived plants registered second highest value for circumference of cormlets in the var. Varada (Tables 37, 38 and 39).

4.5.1.9. Number of nodes per cormlets

Analysis of variance for the years of 2003-04 and 2005-06 revealed that the effects of explants and variety X explant interaction were significant for the number of nodes per cormlets but the varietal effect was found non significant. However, during the year of 2004-05, the effects of variety, explant and variety X explant interaction were found significant (Table 42).

Table 42. Analysis of variance for number of nodes per cormlets in field grown tissue cultured plants.

Source	Degrees of freedom	2003-2004			2004-2005			2005-2006		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Variety	1	0.98	0.98	2.27^{ns}	26.62	26.62	55.86**	0.03	0.03	0.09^{ns}
Explant	3	33.08	11.03	25.51**	43.16	14.39	30.19**	21.00	7.00	18.53**
Variety X Explant	3	9.69	3.23	7.48**	23.69	7.90	16.57**	3.46	1.15	3.05*
Error	72	31.12	0.43		34.31	0.48		27.20	0.38	
Total	79	74.87			127.79			51.70		

Mean values of number of nodes per cormlets recorded from the field grown ginger plants, during three years of study are given in the Tables 37, 38 and 39. Among the two varieties studied, the var. Jamaica showed maximum number of nodes per cormlets as compared to the var. Varada in all the three years of study.

In the var. Jamaica, the number of nodes per cormlet were more in the control plants followed by the plants regenerated from vegetative bud in 2003-04 and 2005-06. But in the year 2004-05, the aerial stem regenerated plants followed by the control plants showed maximum number of nodes per cormlet. However, in the var. Varada, the control plants showed maximum number of nodes per cormlets followed by the aerial stem regenerated plantlets in all the years. Minimum number of nodes per cormlets was observed in the callus regenerated plantlets in both the varieties during the three years (Tables 37, 38 and 39).

4.5.1.10. Length of internodes

Analysis of variance for internodal length of cormlets revealed that, the length of internodes was not significantly influenced by varieties and explants in the year 2003-04. The interaction between variety X explant was also non significant (Table 43). In the year 2004-05, the effects of explants and variety X explant interaction were significant, while the varietal effect was non significant (Table 43). In the year 2005-06, the interaction effect of variety X explant was significant but the effects of varieties and explants were not significant (Table 43).

Table 43. Analysis of variance for internodal length of cormlets in field grown tissue cultured plants.

Source	Degrees of freedom	2003-2004			2004-2005			2005-2006		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Variety	1	0.41	0.41	1.55^{ns}	0.03	0.03	3.64^{ns}	0.00	0.00	0.15^{ns}
Explant	3	0.44	0.15	0.55^{ns}	0.28	0.09	11.12**	0.09	0.03	2.41^{ns}
Variety X Explant	3	0.81	0.27	1.02^{ns}	0.16	0.05	6.32**	0.14	0.05	3.93*
Error	72	19.01	0.26		0.60	0.01		0.88	0.01	
Total	79	20.67			1.06			1.11		

The mean values of internodal length of cormlets recorded from the field grown ginger plants, during three years of study are given in the Tables 37, 38 and 39. The results showed that the var. Varada showed maximum length of internodes per cormlets during the year 2003-2004, while during the years 2004-2005 and 2005-2006, both the varieties showed approximately same internodal length (Tables 37, 38 and 39).

The var. Jamaica showed maximum internodal length in the control plants followed by the plants regenerated from aerial stem and vegetative buds in the year 2003-04. In 2004-05, the control plants and the vegetative bud regenerated plants had maximum internodal length. In the year (2005-06) however, the vegetative bud regenerated plants showed maximum internodal length followed by the aerial stem regenerated plants. The callus regenerated plants showed minimum internodal length in all the 3 years of study (Tables 37, 38 and 39).

In the var. Varada, the callus regenerated plants registered maximum internodal length followed by the control plants and minimum in the vegetative bud regenerated plants in the year 2003-04. In 2004-05, the control plants showed maximum internodal length followed by the aerial stem regenerated plants. Minimum length was observed in the plants regenerated from vegetative bud. In the year 2005-06 the control plants followed by the callus regenerated plants showed maximum

internodal length and minimum was observed in the plants regenerated from aerial stem and vegetative bud (Tables 37, 38 and 39).

4.5.1.11. Weight of rhizome

Effects of explants and variety X explant interaction were found significant for weight of rhizome in all the three years of study. However, the varietal effect was significant only in the year 2004-05 (Table 44).

Table 44. Analysis of variance for rhizome weight in field grown tissue cultured plants.

Source	Degrees of freedom	2003-2004			2004-2005			2005-2006		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Variety	1	84.26	84.26	1.501 ^{ns}	227.48	227.48	5.56*	139.13	139.13	1.79 ^{ns}
Explant	3	44661.7	14887.2	265.12**	45286.4	15095.49	369.14**	59607.9	19869.28	255.07**
Variety X Explant	3	2083.17	694.39	12.37**	2736.05	912.02	22.30**	1806.83	602.28	7.73**
Error	72	4043.03	56.15		2944.35	40.90		5608.73	77.90	
Total	79	50872.1			51194.3			67162.6		

Mean values of rhizome weight recorded from the field grown ginger plants, during three years of study are given in the Tables 37, 38 and 39. The results indicated that the var. Jamaica produced more rhizomes as compared to the var. Varada.

The maximum weight of rhizome was observed in the control plants followed by the aerial stem regenerated plants and the minimum rhizome weight was observed in the callus regenerated plants in both the varieties (Tables 37, 38 and 39).

4.5.2. Pooled data – Morphological and yield characters.

4.5.2.1. Plant height

Analysis of variance for pooled data of plant height revealed that the effects of year, variety, explant, year X explant (Y x E), variety X explant (V x E) and year X

variety X explant (Y x V x E) interactions were significant for plant height (Table 45). The interaction between year X variety was found non significant indicating the stable performance of the varieties over the years.

Table 45. Analysis of variance for plant height - pooled data).

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Year	2	1400.7	700.4	37.76**
Variety	1	1542.8	1542.8	83.18**
Year X Variety	2	58.4	29.2	1.58^{ns}
Explant	3	5960.9	1987.0	107.13**
Year X Explant	6	1732.9	288.8	15.57**
Variety X Explant	3	1534.7	511.6	27.58**
Year X Variety X Explant	6	439.4	73.2	3.95**
Error	216	4006.2	18.6	
Total	239	16676.1		

Mean values of plant height recorded from field grown ginger plants, during three years of study are presented in the Table 46. Among the two varieties studied, the var. Varada exhibited maximum plant height (67.55 cm) as compared to the var. Jamaica (62.48 cm).

Table 46. Mean values of morphological characters recorded from field grown ginger plants (pooled data).

Treatment	Plant height (cm)			No. of tiller			No. of leaves			Leaf length (cm)			Leaf width (cm)		
	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean
1	69.23	72.89	71.06	12.97	12.37	12.67	20.70	20.10	20.4	21.16	21.70	21.36	2.55	2.71	2.63
2	62.98	64.52	63.75	9.67	9.400	9.54	18.96	18.80	18.88	21.09	20.34	20.72	2.61	2.56	2.58
3	56.93	58.33	57.63	8.13	7.967	8.05	15.70	16.76	16.23	19.29	18.50	18.90	2.40	2.37	2.38
4	60.76	74.45	67.61	7.23	7.433	7.33	17.93	19.13	18.53	19.10	23.52	21.31	2.77	2.86	2.82
Mean	62.48	67.55		9.5	9.3		18.32	18.70		20.16	21.0		2.58	2.63	
CD (P=0.05)															
Year	1.36			NS			0.67			0.34			0.06		
Variety	1.11			NS			NS			0.27			NS		
Year X Variety	NS			NS			NS			0.95			NS		
Explant	1.57			0.82			0.78			0.39			0.06		
Year X Explant	3.84			2.00			1.9			NS			0.16		
Variety X Explant	3.84			NS			1.9			0.95			0.16		
Y x V x E	3.84			NS			NS			NS			NS		
CV (%)	6.62			NS			11.5			5.2			6.7		

Treatments: 1) Aerial stem regenerated plants, 2) Vegetative bud regenerated plants, 3) Callus regenerated plants, 4) Control plants.

In the var. Jamaica, the aerial stem derived plants showed maximum plant height followed by the vegetative bud regenerated plants and the control plants. However, in the var. Varada, the control plants followed by the aerial stem regenerated plants and the vegetative bud derived plants exhibited maximum plant height. Minimum plant height was recorded from the callus regenerated plants in both the varieties (Table 46).

4.5.2.2. Number of tillers

Statistical analysis of pooled data for tiller number revealed significant effects of explants and year X explant interaction for number of tillers per plant. Eventhough the tiller number varied significantly with explants it did not vary with the different varieties in the different years (Table 47).

Table 47. Analysis of variance for number of tillers - (pooled data).

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Year	2	1.23	0.62	0.123 ^{ns}
Variety	1	2.60	2.60	0.518 ^{ns}
Year X Variety	2	18.63	9.32	1.853 ^{ns}
Explant	3	1006.95	335.65	66.753**
Year X Explant	6	117.17	19.53	3.884**
Variety X Explant	3	4.88	1.63	0.324 ^{ns}
Year X Variety X Explant	6	35.83	5.97	1.188 ^{ns}
Error	216	1086.10	5.03	
Total	239	2273.40		

Mean values of number of tillers recorded from field grown ginger plants, during three years of study are presented in the Table 46. The mean values of tiller number for varieties showed that the var. Jamaica (9.5) produced slightly more number of tillers compared to the var. Varada (9.3). Among the explants tried, the aerial stem regenerated plants followed by the vegetative bud derived plants exhibited maximum number of tillers and minimum number of tillers were produced by the control plants in both the varieties (Table 46).

4.5.2.3. Number of leaves

Analysis of variance of pooled data for number of leaves revealed significant effects of explants, years, year X explant and variety X explant interactions. However, the effects of variety, year X variety and year X variety X explant interactions were found non significant (Table 48).

Table 48. Analysis of variance for number of leaves - (pooled data).

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Year	2	131.58	65.79	14.48**
Variety	1	8.44	8.44	1.86^{ns}
Year X Variety	2	5.48	2.74	0.60^{ns}
Explant	3	533.71	177.90	39.16**
Year X Explant	6	82.53	13.75	3.03**
Variety X Explant	3	36.05	12.02	2.65*
Year X Variety X Explant	6	8.89	1.48	0.33^{ns}
Error	216	981.30	4.54	
Total	239	1787.96		

The significant interaction effects indicate that the performance of the explants is influenced by the environmental factors of the different years as well as the varieties. This implies that the explant from a particular genotype can exhibit better performance in a particular environment.

Mean values of number of leaves recorded from field grown ginger plants, during three years of study are presented in the Table 46. The results showed that both the varieties were at par for mean leaf number (Table 46).

Among the explants tried, the aerial stem regenerated plants followed by the vegetative bud derived plants exhibited maximum number of leaves in the var. Jamaica. However, in the var. Varada, the aerial stem derived plants followed by the control plants showed maximum number of leaves. Minimum number of leaves recorded from the callus regenerated plants in both the varieties (Table 46).

4.5.2.4. Leaf length

Analysis of variance of pooled data for leaf length showed that leaf length varied significantly with varieties and explants in the different years crop. The interactions between year X variety and variety X explant were highly significant for leaf length, whereas the year X explant interaction and year X variety X explant interaction were found non significant (Table 49).

Table 49. Analysis of variance for length of leaves - (pooled data).

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Year	2	12.53	6.27	5.57**
Variety	1	42.21	42.21	37.48**
Year X Variety	2	12.26	6.13	5.44**
Explant	3	236.27	78.76	69.94**
Year X Explant	6	7.18	1.20	1.06^{ns}
Variety X Explant	3	271.44	90.48	80.35**
Year X Variety X Explant	6	13.60	2.27	2.01^{ns}
Error	216	243.23	1.13	
Total	239	838.72		

Mean values of leaf length recorded from field grown ginger plants, during three years of study are given in the Table 46. Among the two varieties studied, the var. Varada showed maximum leaf length (21.0 cm) as compared to the var. Jamaica (20.16 cm). Varietal differences were observed in the leaf length of plants with different explants used for the study. In the var. Jamaica, the aerial stem regenerated followed by the vegetative bud derived plants exhibited maximum leaf length. The control plants registered minimum leaf length. However, in the var. Varada, the control plants followed by the aerial stem regenerated plants exhibited maximum leaf length and the callus regenerated plants showed minimum leaf length (Table 46).

4.5.2.5. Leaf width

Analysis of variance for leaf width indicates significant effects of explant, year, year X explant and variety X explant interactions. However, the leaf width did

not significantly vary with different varieties. The effects of year X variety and year X variety X explant interactions were also non significant (Table 50).

Table 50. Analysis of variance for width of leaves - (pooled data).

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Year	2	0.47	0.24	7.71**
Variety	1	0.12	0.12	3.74^{ns}
Year X Variety	2	0.03	0.02	0.54^{ns}
Explant	3	5.59	1.86	60.69**
Year X Explant	6	1.08	0.18	5.87**
Variety X Explant	3	0.44	0.15	4.75**
Year X Variety X Explant	6	0.28	0.05	1.54^{ns}
Error	216	6.63	0.03	
Total	239	14.64		

Mean values of leaf width recorded from field grown ginger plants, during three years of study are presented in the Table 46. In this case both the varieties showed an average leaf width of 2.6 cm (Table 46). In the different explant sources tried, the control plants followed by the vegetative bud derived plants exhibited maximum leaf width in the var. Jamaica. However, in the var. Varada, the control plants followed by the aerial stem regenerated plants had maximum leaf width. Minimum leaf width was recorded from the callus regenerated plants in both the varieties (Table 46).

4.5.2.6. Number of cormlets

The number of cormlets was significantly influenced by the year of cultivation and type of explant from which the plants were regenerated. The variety X explant interaction was also found significant. The effects of variety, year X variety, year X explant and year X variety X explant interactions for the number of cormlets were non significant (Table 51).

Table 51. Analysis of variance for number of cormlets - (pooled data).

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Year	2	72.03	36.02	6.88**
Variety	1	0.20	0.20	0.04^{ns}
Year X Variety	2	12.43	6.22	1.19^{ns}
Explant	3	4147.95	1382.65	263.99**
Year X Explant	6	60.07	10.01	1.91^{ns}
Variety X Explant	3	91.78	30.59	5.84**
Year X Variety X Explant	6	31.73	5.29	1.01^{ns}
Error	216	1131.30	5.24	
Total	239	5547.50		

Mean values of number of cormlets recorded from field grown ginger plants, during three years of study are presented in the Table 52. Mean values of number of cormlets for varieties revealed that the var. Varada and the var. Jamaica produced approximately same number of cormlets (11.4 and 11.5, respectively). Among the explants tried, the control plants followed by the aerial stem regenerated plants showed maximum number of cormlets and minimum number of cormlets was observed with the callus regenerated plants in both the varieties (Table 52).

Table 52. Mean values of yield attributes recorded from field grown ginger plants (pooled data).

Treatment	No. of cormlets			Length of cormlets (cm)			Circumference of cormlets (cm)			No. of nodes/cormlet			Length of inter nodes (cm)			Weight of rhizomes (g)		
	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean
1	14.9	13.9	14.4	3.57	2.42	2.30	6.54	4.35	5.45	6.16	5.42	5.79	0.54	0.50	0.52	54.7	41.96	48.3
2	10.9	10.4	10.6	2.82	2.24	2.53	5.30	4.01	4.66	6.11	5.18	5.65	0.55	0.45	0.5	44.7	37.12	40.9
3	3.90	6.10	5.02	1.99	1.90	1.95	3.19	3.80	3.50	4.28	4.87	4.58	0.46	0.49	0.48	16.1	12.93	14.5
4	16.0	15.5	15.7	3.95	4.17	4.06	6.95	7.51	7.23	6.65	5.84	6.25	0.57	0.64	0.61	77.2	91.26	84.2
Mean	11.4	11.5		3.1	2.7		5.5	4.9		5.8	5.3		0.53	0.52		48.2	45.8	
CD (P=0.05)																		
Year	0.72			NS			NS			0.21			0.032			2.3		
Variety	NS			0.13			0.14			0.17			NS			1.9		
Year X Variety	NS			NS			NS			0.58			NS			NS		
Explant	0.83			0.18			0.19			0.24			0.036			2.7		
Year X Explant	NS			NS			NS			0.58			NS			6.5		
Variety X Explant	2.04			0.45			0.47			0.58			0.089			6.5		
Y x V x E	NS			NS			0.47			0.58			NS			NS		
CV (%)	20.0			17.5			10.1			11.8			18.8			15.5		

Treatments: 1) Aerial stem regenerated plants, 2) Vegetative bud regenerated plants, 3) Callus regenerated plants, 4) Control plants.

4.5.2.7. Length of cormlets

The effects of variety, explant and variety X explant interaction for the length of cormlets were significant. However, the cormlet length did not vary significantly with the years of planting. The interactions between year X variety, year X explant and year X variety X explant were non significant (Table 53).

Table 53. Analysis of variance for length of cormlets - (pooled data).

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Year	2	1.36	0.68	2.66^{ns}
Variety	1	9.70	9.70	38.06**
Year X Variety	2	0.61	0.31	1.21^{ns}
Explant	3	144.12	48.04	188.56**
Year X Explant	6	1.64	0.27	1.07^{ns}
Variety X Explant	3	15.96	5.32	20.89**
Year X Variety X Explant	6	0.99	0.16	0.65^{ns}
Error	216	55.03	0.26	
Total	239	229.40		

Mean values of length of cormlets recorded from field grown ginger plants, during three years of study are presented in the Table 52. The results indicated that the var. Jamaica showed longer cormlets (3.1cm) as compared to the var. Varada (2.7 cm). Among the explants tried, the control plants followed by the aerial stem regenerated plants showed maximum length of cormlets. Minimum length of cormlets were observed with the callus regenerated plants in both the varieties (Table 52).

4.5.2.8. Circumference of cormlets

Circumference of cormlets varied significantly with different varieties and explants. The interactions between variety X explant and year X variety X explant were also found significant, while the circumference of cormlets showed no significant difference with the years of cultivation. The effects of year X explant and year X variety interactions were also non significant (Table 54).

Table 54. Analysis of variance for circumference of cormlets - (pooled data).

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Year	2	1.17	0.58	2.12^{ns}
Variety	1	20.10	20.10	73.21**
Year X Variety	2	1.23	0.61	2.24^{ns}
Explant	3	443.67	147.89	538.74**
Year X Explant	6	0.68	0.11	0.41^{ns}
Variety X Explant	3	87.03	29.01	105.68**
Year X Variety X Explant	6	4.19	0.70	2.54*
Error	216	59.30	0.28	
Total	239	617.36		

Mean values of circumference of cormlets recorded from field grown ginger plants, during three years of study are given in the Table 52. Mean values for varieties showed that the var. Jamaica exhibited maximum circumference of cormlets (5.5 cm) compared to the var. Varada (4.9 cm). Among the explants tried, the control plants followed by the aerial stem derived plants produced maximum circumference of cormlets and minimum circumference of cormlets were observed with the callus regenerated plants in both the varieties (Table 52).

4.5.2.9. Number of nodes per cormlet

Number of nodes per cormlets were significantly influenced by the years of cultivation, varieties and explants used in the study. The effects of year X variety, year X explant, variety X explant and year X variety X explant interactions for number of nodes were also significant (Table 55).

Table 55. Analysis of variance for number of nodes per cormlet - (pooled data).

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Year	2	10.32	5.16	12.03**
Variety	1	13.37	13.37	31.17**
Year X Variety	2	14.27	7.13	16.64**
Explant	3	89.92	29.97	69.89**
Year X Explant	6	7.33	1.22	2.85*
Variety X Explant	3	23.11	7.70	17.96**
Year X Variety X Explant	6	13.73	2.29	5.34**
Error	216	92.64	0.43	
Total	239	264.68		

Mean values of number of nodes per cormlets recorded from field grown ginger plants, during three years of study are presented in the Table 52. The results showed that maximum number of nodes were observed in the var. Jamaica (5.8) as compared to the var. Varada (5.3). Among the explants tried, the control plants followed by the aerial stem derived plants produced maximum number of nodes per cormlets, while minimum number of nodes per cormlets were observed in the callus regenerated plants in both the varieties (Table 52).

4.5.2.10. Internodal length

Internodal length varied significantly with years of cultivation and type of explants. The interaction between variety X explant was also found significant. The effects of variety, year X variety, year X explant and year X variety X explant interactions were non significant (Table 56).

Table 56. Analysis of variance for internodal length - (pooled data).

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Year	2	0.07	0.04	3.77*
Variety	1	0.00	0.00	0.21 ^{ns}
Year X Variety	2	0.05	0.02	2.53 ^{ns}
Explant	3	0.58	0.19	20.08**
Year X Explant	6	0.09	0.02	1.57 ^{ns}
Variety X Explant	3	0.25	0.08	8.58**
Year X Variety X Explant	6	0.07	0.01	1.22 ^{ns}
Error	216	2.08	0.01	
Total	239	3.19		

Mean values of internodal length per cormlets recorded from field grown ginger plants, during three years of study are presented in the Table 52. Internodal length was approximately same for the two varieties studied (var. Jamaica 0.53 cm and var. Varada 0.52 cm). The explants used for plant regeneration had high influence in this case. The control plants followed by the vegetative bud regenerated plants showed maximum length of internodes and minimum internodal length was recorded from the callus regenerated plants in the var. Jamaica. However, in the var.

Varada, the control plants followed by the aerial stem derived plants showed maximum internodal length and minimum internodal length was observed in the vegetative bud derived plants (Table 52).

4.5.2.11. Weight of rhizome

The rhizome weight was significantly influenced by the effects of year, variety, explant, year X explant and variety X explant interactions. However, the year X variety and year X variety X explant interactions were found non significant (Table 57).

Table 57. Analysis of variance for weight of rhizome - (pooled data).

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Year	2	635.58	317.79	5.97**
Variety	1	332.53	332.53	6.25*
Year X Variety	2	56.23	28.12	0.53 ns
Explant	3	148715.91	49571.97	931.08**
Year X Explant	6	932.37	155.40	2.92**
Variety X Explant	3	6103.19	2034.40	38.21**
Year X Variety X Explant	6	186.56	31.09	0.58 ns
Error	216	11500.10	53.24	
Total	239	168462.47		

Mean values of rhizome weight recorded from field grown ginger plants, during three years of study are given in the Table 52. The results indicated that the var. Jamaica produced more rhizomes (48.2 g) as compared to the var. Varada (45.8 g). Among the explants tried, the control plants followed by the aerial stem regenerated plants showed maximum weight of rhizomes and the minimum rhizome weight was observed with the callus regenerated plants in both the varieties (Table 52).

4.5.3. Field evaluation of second generation plants

4.5.3.1. Plant height

Analysis of variance for plant height revealed significant effects of variety, explant and variety X explant interaction for plant height (Table 58).

Table 58. Analysis of variance for plant height – second generation.

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Variety	1	781.88	781.88	52.27**
Explant	3	5488.18	1829.39	122.31**
Variety X Explant	3	466.19	155.39	10.39**
Error	72	1076.95	14.96	
Total	79	7813.18		

Mean values of plant height recorded from the second generation ginger plants are presented in the Table 59. The mean values of plant height for varieties indicated that the var. Varada showed maximum plant height (67.4 cm) as compared to the var. Jamaica (61.1 cm). In the var. Varada, the control plants showed maximum plant height followed by the aerial stem regenerated plants. In the var. Jamaica, the aerial stem regenerated plants showed maximum plant height followed by the vegetative bud derived plants. Minimum plant height was observed in the callus regenerated plants in both the varieties (Table 59).

Table 59. Mean values of morphological characters recorded from the second generation ginger plants.

Treatment	Plant height (cm)			No. of tiller			No. of leaves			Leaf length (cm)			Leaf width (cm)		
	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean
1	68.3	69.9	69.1	9.3	7.5	8.4	20.5	22.1	21.3	19.0	22.8	20.9	2.9	2.8	2.85
2	66.5	69.6	68.05	8.5	7.8	8.15	19.7	20.3	20	20.1	21.5	20.8	2.8	2.7	2.75
3	46.8	53.1	49.95	5.4	6.4	5.9	15.1	18.9	17	18.9	20.1	19.5	2.2	2.6	2.40
4	62.8	76.9	69.85	8.2	8.5	8.35	19.7	20.8	20.25	19.6	23.6	21.6	2.8	3.0	2.90
Mean	61.1	67.4		7.85	7.55		18.8	20.5		19.4	22.0		2.67	2.78	
CD (P=0.05)															
Variety	1.73			NS			0.84			0.65			0.08		
Explant	2.45			0.88			1.19			0.92			0.11		
Variety X Explant	3.47			1.24			1.69			1.30			0.16		
CV (%)	6.02			18.0			9.59			6.97			6.38		

Treatments: 1) Aerial stem regenerated plants 2) Vegetative bud regenerated plants 3) Callus regenerated plants 4) Control plants.

4.5.3.2. Tiller number

Total number of tillers varied significantly with the type of explants from which the rhizomes were evolved (planting material for the study). The interaction between variety X explant was significant but the varietal influence was non significant. (Table 60).

Table 60. Analysis of variance for number of tillers – second generation.

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Variety	1	1.80	1.80	0.94^{ns}
Explant	3	87.10	29.03	15.19**
Variety X Explant	3	22.30	7.43	3.89*
Error	72	137.60	1.91	
Total	79	248.80		

Mean values of tiller number recorded from the second generation ginger plants are presented in the Table 59. Among the two varieties studied, the var. Jamaica produced more number of tillers (7.85) when compared to the var. Varada (7.55). In the var. Jamaica, maximum number of tillers were observed in the aerial stem derived plants followed by the vegetative bud regenerated plants, whereas in the var. Varada, maximum tiller number were observed in the control plants followed by the vegetative bud regenerated plants. Minimum tiller number observed in the callus regenerated plants in both the varieties (Table 59).

4.5.3.3. Number of leaves

Number of leaves varied significantly with varieties and type of explant used for plant propagation in the first generation. The interaction between variety X explant interaction was also significant (Table 61).

Table 61. Analysis of variance for number of leaves – second generation.

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Variety	1	63.01	63.01	17.79**
Explant	3	204.53	68.18	19.24**
Variety X Explant	3	29.84	9.95	2.81*
Error	72	255.10	3.54	
Total	79	552.49		

Mean values of number of leaves recorded from second generation ginger plants are presented in the Table 59. Among the two varieties studied the var. Varada produced more number of leaves (20.5) as compared to the var. Jamaica (18.8).

Maximum leaf number was observed in the aerial stem regenerated plants followed by the control plants and minimum number of leaves was observed in the callus regenerated plants in both the varieties (Table 59).

4.5.3.4. Leaf length

The leaf length varied significantly with varieties and explants used for plant propagation. The interaction between variety X explant was also found significant (Table 62).

Table 62. Analysis of variance for length of leaves – second generation.

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Variety	1	136.77	136.77	65.49**
Explant	3	46.27	15.42	7.39**
Variety X Explant	3	32.47	10.82	5.18**
Error	72	150.35	2.09	
Total	79	365.86		

Mean values of leaf length recorded from the second generation ginger plants are presented in the Table 59. Mean number of leaf length for varieties revealed that the var. Varada showed maximum leaf length (22.0 cm) as compared to the var. Jamaica (19.4 cm). In the var. Jamaica, maximum leaf length was observed in the

vegetative bud derived plants followed by the control plants. In the var. Varada, maximum leaf length was in the control plants followed by the aerial stem derived plants. Minimum leaf length was observed with the callus regenerated plants in both the varieties (Table 59).

4.5.3.5. Leaf width

Analysis of variance for leaf width revealed significant effects of varieties, explants and variety X explant interaction for leaf width of ginger (Table 63).

Table 63. Analysis of variance for width of leaves – second generation.

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Variety	1	0.14	0.14	4.75*
Explant	3	3.08	1.03	33.88**
Variety X Explant	3	0.67	0.22	7.38**
Error	72	2.18	0.03	
Total	79	6.07		

Mean values of leaf width recorded from the second generation crop are presented in the Table 59. Among the two varieties, the var. Varada (2.78 cm) showed more leaf width as compared to the var. Jamaica (2.67 cm).

In the var. Jamaica, maximum leaf width was observed in the aerial stem derived plants followed by the control plants and vegetative bud regenerated plants. However, in the var. Varada, the control plants followed by aerial stem derived plants had maximum leaf width. Minimum leaf width was observed in the callus regenerated plants in both the varieties (Table 59).

4.5.3.6. Number of cormlets

Analysis of variance for number of cormlets revealed the significant effects of varieties and explants for number of cormlets but the interaction between variety X explant was non significant (Table 64).

Table 64. Analysis of variance for number of cormlets – second generation.

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Variety	1	99.01	99.01	13.57**
Explant	3	1568.44	522.81	71.63**
Variety X Explant	3	22.74	7.58	1.04 ns
Error	72	525.50	7.30	
Total	79	2215.69		

Mean values of number of cormlets recorded from the second generation crop are presented in the Table 65. The results showed that the var. Jamaica (14.7) produced more number of cormlets as compared to the var. Varada (12.5). In both the varieties the aerial stem regenerated plants showed maximum number of cormlets followed by the control plants and the callus regenerated plants showed minimum number of cormlets (Table 65).

Table 65. Mean values of yield attributes recorded from the second generation ginger plants.

Treatment	No. of cormlets			Length of cormlets (cm)			Circumference of cormlets (cm)			No. of nodes/cormlet			Length of inter nodes (cm)			Weight of rhizomes (g)		
	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean
1	19.4	16.3	17.85	3.1	4.31	3.70	7.9	7.3	7.60	7.0	6.7	6.9	0.5	0.6	0.55	157.0	76.4	116.7
2	13.0	10.3	11.65	2.7	3.06	2.88	6.4	7.1	6.75	6.9	5.7	6.3	0.6	0.5	0.55	109.7	59.6	84.65
3	7.4	7.0	7.20	2.1	2.34	2.22	3.3	4.2	3.75	4.8	4.5	4.7	0.47	0.4	0.44	28.1	24.2	26.15
4	18.9	16.2	17.55	4.3	4.38	4.34	6.8	8.1	7.45	5.9	5.9	5.9	0.5	0.6	0.55	84.1	96.8	90.45
Mean	14.7	12.5		3.05	3.52		6.1	6.7		6.2	5.7		0.52	0.53		94.7	64.3	
CD (P=0.05)																		
Variety	1.21			0.22			0.27			0.35			NS			5.56		
Explant	1.71			0.31			0.38			0.5			0.08			7.86		
Variety X Explant	NS			0.44			0.54			NS			0.11			11.1		
CV (%)	19.9			14.8			9.45			13.3			23.4			15.6		

Treatments: 1) Aerial stem regenerated plants 2) Vegetative bud regenerated plants 3) Callus regenerated plants 4) Control plants.

4.5.3.7. Length of cormlets

Analysis of variance revealed the significant effects of varieties, explant and variety X explant interaction for length of cormlets (Table 66).

Table 66. Analysis of variance for length of cormlets – second generation.

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Variety	1	4.06	4.06	16.92**
Explant	3	49.67	16.56	69.03**
Variety X Explant	3	5.18	1.73	7.20**
Error	72	17.27	0.24	
Total	79	76.18		

Mean values of length of cormlets recorded from the second generation ginger plants are presented in the Table 65. Mean values for length of cormlets for varieties indicated that the var. Varada showed maximum cormlet length (3.52 cm) compared to the var. Jamaica (3.05 cm). The mean values for treatments revealed that the control plants had maximum length of cormlets followed by the aerial stem derived plants. The callus regenerated plants showed minimum length of cormlets in both the varieties (Table 65).

4.5.3.8. Circumference of cormlets

Circumference of cormlets varied significantly with varieties and explants. The interaction between variety X explant was also significant (Table 67).

Table 67. Analysis of variance for circumference of cormlets – second generation.

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Variety	1	6.92	6.92	19.067**
Explant	3	189.79	63.26	174.29**
Variety X Explant	3	9.60	3.20	8.82**
Error	72	26.13	0.36	
Total	79	232.45		

Mean values of circumference of cormlets recorded from the second generation ginger plants are presented in the Table 65. The results indicated that the var. Varada (6.7 cm) showed maximum circumference of cormlets as compared to the var. Jamaica (6.1 cm).

The mean values for treatments in the var. Jamaica revealed that, the aerial stem regenerated plants followed by the control plants showed maximum circumference of cormlets. However, in the var. Varada, the control plants followed by the aerial stem derived plants had maximum circumference of cormlets. Minimum circumference of cormlets was recorded from the callus regenerated plants in both the varieties (Table 65).

4.5.3.9. Number of nodes per cormlets

Number of nodes per cormlets varied significantly with varieties and explants. But the variety X explant interaction was non significant (Table 68).

Table 68. Analysis of variance for number nodes – second generation.

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Variety	1	3.42	3.42	5.50*
Explant	3	54.97	18.32	29.45**
Variety X Explant	3	4.24	1.41	2.27^{ns}
Error	72	44.79	0.62	
Total	79	107.42		

Mean values of number of nodes per cormlets recorded from the second generation ginger plants are presented in the Table 65. The results showed that the var. Jamaica had more number of nodes per cormlets (6.2) as compared to the var. Varada (5.7). The mean number for treatments revealed that the aerial stem derived plants followed by the vegetative bud regenerated plants produced more number of nodes per cormlet in the var. Jamaica. In the var. Varada, the aerial stem derived plants followed by the control plants produced maximum number of nodes per

cormlet. The callus regenerated plants exhibited minimum number of nodes per cormlets in both the varieties (Table 65).

4.5.3.10. Length of internodes

Length of internodes varied significantly with the explants used for plant regeneration. The interaction of variety X explant was also found significant but the varietal effect was not significant (Table 69).

Table 69. Analysis of variance for length of internodes – second generation.

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Variety	1	0.00	0.00	0.07^{ns}
Explant	3	0.27	0.09	6.01**
Variety X Explant	3	0.16	0.05	3.58*
Error	72	1.09	0.02	
Total	79	1.53		

Mean values of length of internodes recorded from the second generation ginger plants are presented in the Table 65. The two varieties produced cormlets of approximately equal internodal length of 0.5 cm. Mean values of internodal length for treatments revealed that the vegetative bud regenerated plants followed by the aerial stem derived and the control plants showed maximum internodal length in the var. Jamaica. In the var. Varada, the aerial stem derived and the control plants showed maximum length of internodes followed by the vegetative bud regenerated plants. In both the varieties the callus regenerated plants showed minimum length of internodes (Table 65).

4.5.3.11. Weight of rhizomes

Analysis of variance for rhizome weight revealed significant effect of varieties, explants and variety X explant interaction for weight of rhizomes (Table 70).

Table 70. Analysis of variance for weight of rhizome – second generation.

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Variety	1	18574.51	18574.51	120.79**
Explant	3	87529.74	29176.58	189.73**
Variety X Explant	3	27339.84	9113.28	59.26**
Error	72	11071.90	153.78	
Total	79	144515.99		

Mean values of rhizome weight recorded from the second generation crop are presented in the Table 65. The results showed that the var. Jamaica produced high yield (94.7 g) as compared to the var. Varada (64.3 g). The mean number for the treatments indicate that the aerial stem derived plants followed by the vegetative bud regenerated plants had maximum weight of rhizomes in the var. Jamaica. In the var. Varada, the control plants followed by the aerial stem derived plants showed maximum weight of rhizomes. In both the varieties the callus regenerated plants recorded least rhizome weight (Table 65).

In the present study, the performance of tiller number, length of cormlets and circumference of cormlets were stable during different years of crop as revealed by the non significant effect of years (Tables 47, 53 & 54) though, Samsudeen (1996) reported year wise variation for almost all characters in micropropagated plants of ginger except tiller number.

Further, the performance of varieties used in the study varied in first and the second generations. The var. Jamaica showed good performance for number of tillers, length of cormlets, circumference of cormlets, number of nodes per cormlet and weight of rhizomes in the first generation. However, during the second generation, this variety exhibited good performance for the characters such as tiller number, number of cormlets, number of nodes per cormlet and weight of rhizomes.

The var. Varada showed better performance for plant height, number of leaves and leaf length in the first generation but during the second generation, this genotype exhibited better performance for plant height, number of leaves, leaf length, leaf width, length of cormlets and circumference of cormlets.

The performance of explants used in the study also varied with the different varieties over generations. In case of the first generation crop of the var. Jamaica, the aerial stem regenerated plants exhibited good performance for aerial morphological characters like plant height, tiller number, number of leaves and leaf length (Fig. 11. e). However, for the rhizome characters, the control plants exhibited good performance. In the second generation crop, the aerial stem regenerated plants showed good performance for most of the characters like plant height, tiller number, number of leaves, leaf width, number of cormlets, circumference of cormlets, number of nodes per cormlet and weight of rhizomes.

In the first generation crop of the var. Varada, the control plants showed better performance for all the characters except tiller number and leaf number. During the second generation crop, the control plants registered good performance for plant height, tiller number, leaf length, leaf width, length of cormlets, circumference of cormlets and weight of rhizomes. For other traits like number of leaves, number of cormlets, and number of nodes per cormlet, the aerial stem regenerated plants exhibited better performance.

Samsudeen (1996) observed that number of tillers and number of leaves in micropropagated plants were less compared to the control plants and the tillers were shorter in height. The plants produced very small rhizomes with tuberous roots. In the present study, number of tillers was high in all the three groups of micropropagated ginger plants compared to the control plants in both the generations in the var. Jamaica, whereas in the var. Varada, the control plants showed highest tiller number in the second generation crop.

Reasons attributed to increase tillering in the micropropagated plants are residual action of cytokinin, rejuvenation and the origin of explant (Marks and Myers 1992; George, 1993a).

In the present study, the control plants exhibited more rhizome yield as compared to the micropropagated plants in the first generation in both the varieties as normally expected (Fig. 11. f – k). However, during the second generation itself, the aerial stem regenerated plants out yielded the control plants, in the var. Jamaica with large and bold rhizomes (Fig. 11. l & m). Rao *et al.* (2000) reported that the yield obtained from the micropropagated plants was more or less comparable to that obtained from the conventionally propagated plants in the first generation, though it took an additional four months and fifteen days to harvest the rhizome. However, in the present study, the micropropagated plants attained the maturity within 200 days, as observed in the conventionally propagated plants, though the rhizome yield was less as compared to the control.

Nirmal Babu (1997) reported that plantlets regenerated from the callus cultures were inferior to the direct regenerated plants with respect to plant size, hardening time, field establishment and rhizome yield. In the present study also, the callus regenerated plants of both the varieties exhibited poor performance for all the characters studied as compared to other plants in both generations (aerial stem derived, vegetative bud regenerated and control plants) (Fig. 11. e). Nirmal Babu (1997) further observed that, in the first generation, the rhizome yield of tissue cultured plants were too less (0.7 - 3.3 g) to harvest and if harvested, the rhizomes dry up and adversely affect the germination capacity in the next generation. Samsudeen (1996) also reported that tissue cultured ginger plants took three crop seasons to produce rhizomes of normal size. However, in the present study, the aerial stem and the vegetative bud regenerated plants in the first generation crops itself produced medium sized, harvestable, rhizomes (Fig. 11. h - k). The callus regenerated plantlets

produced small rhizomes and showed variations in the rhizome shape also (Fig. 11. n & o).

The *in vitro* regenerated plants were not affected by any of the diseases, which are commonly found in ginger crop probably due to the healthy explants used. Sharma and Singh (1997) reported that the micropropagated ginger plants were morphologically identical to the mother plants and were free of ginger yellows disease.

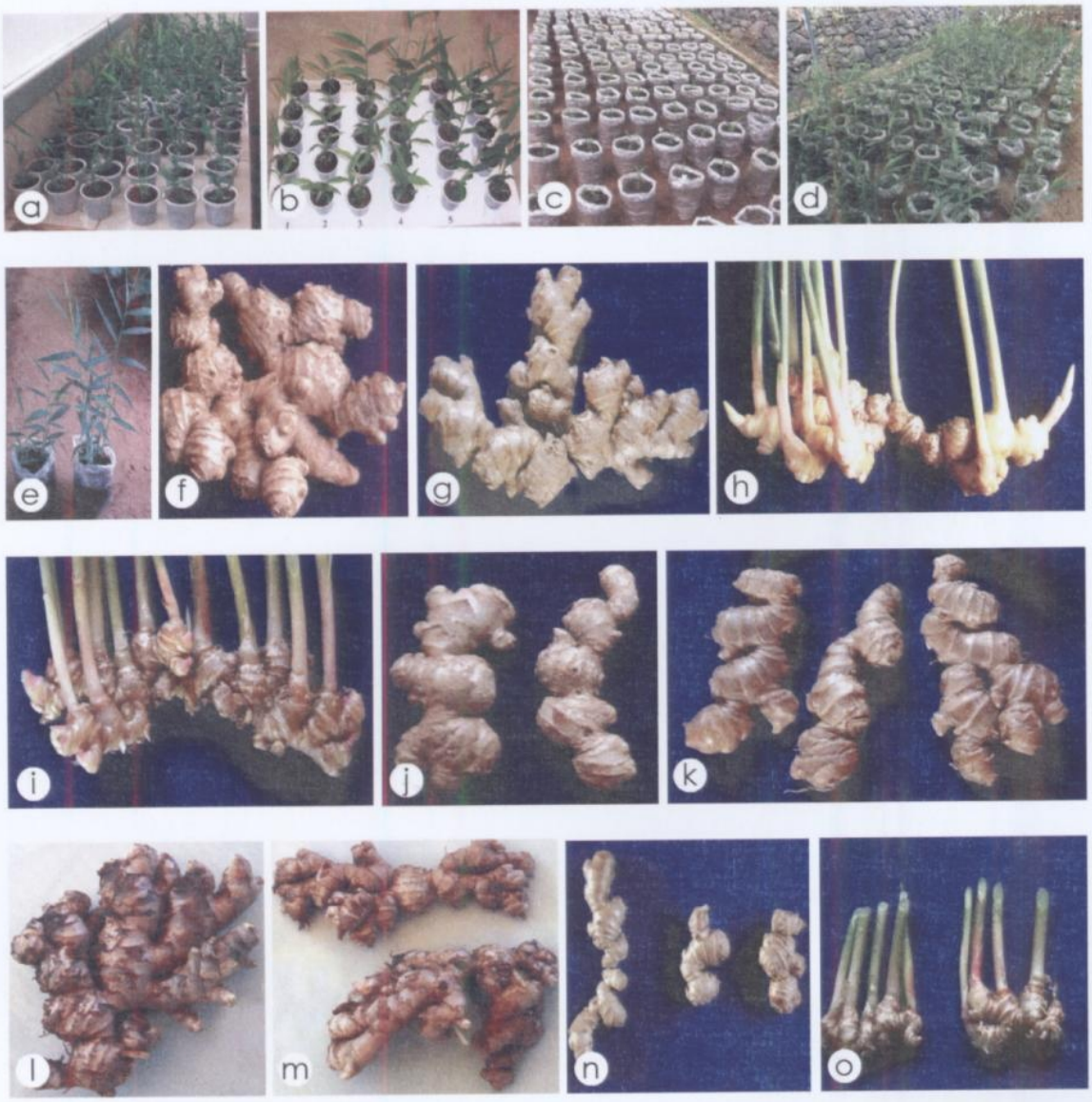


Fig. 11. a). Acclimatization of plantlets. b). Plantlets grown in different hardening medium. c). Hardened plantlets transferred to polythene bags (30 x 40 cm). d). Micropropagated plants grown in the experimental shed (4 months old). e). Aerial stem regenerated plants showing better performance in plant height, tiller number and leaf number as compared to callus regenerated plants (4 months old). f & g). Rhizomes of control plant of var. Jamaica and var. Varada. h & i) Rhizomes of aerial stem regenerated plants of var. Jamaica and var. Varada (first generation). j & k). Rhizomes of vegetative bud regenerated plants of var. Jamaica and var. Varada (First generation). l & m). Rhizomes of aerial stem regenerated plants of var. Jamaica and var. Varada (second generation). n & o). Rhizomes of callus regenerated plants of var. Varada (first and second generation).

Freitez *et al.* (2003) reported significant differences for the morphological and yield characters with the type of propagation (*in vitro* and conventional) and conditions of light (full sun light and partial shade) in ginger. The number of shoots, fresh and dry mass of shoots were higher in *in vitro* propagated plants, which were shorter than those propagated by rhizomes. High rhizome mass was observed in the plants propagated conventionally but the root mass was low as compared to the *in vitro* propagated plants. The *in vitro* plants produced numerous small rhizomes with a high number of fleshy roots with tuberous structures at the tips. This type of rhizomes with fleshy roots and tubers were observed in the present study also.

Explants are known to influence the field performance of the micropropagated plants. The developmental stage of the tissue, physiological age of explants etc. are known variables affecting the performance of the tissue cultured plants (George, 1993b). In the conventionally propagated crop also the standard planting unit is 15 - 20 g rhizome bits with 1-3 viable buds (Nybe and Raj, 2005). Planting unit below this size are known to affect yield.

4.5.4. Yield evaluation of plants grown in large beds

Mean values of characters observed from the plants grown in large bed (non replicated) under field condition and under 50 % shade (in the experimental shed) are given in the Table 71.

Table 71. Comparative performance of tissue cultured (aerial stem regenerated) ginger plants grown in large beds with and without shade.

Variety	Growing condition	Plant height (cm)	No. of tiller	No. of leaves	Leaf length (cm)	Leaf width (cm)	No. of cormlets	Length of cormlets (cm)	Circum. of cormlets (cm)	No. of nodes/ cormlet	Length of internodes (cm)	Weight of rhizomes (g)
Jamaica	Field	56.2	12.3	16.2	17.9	2.33	15.0	2.38	4.28	5.16	0.47	42.4
	Shade	75.6	11.6	20.8	21.7	2.74	14.6	2.43	4.40	5.46	0.43	44.4

Characters such as plant height, number of leaves, leaf length, leaf width, length of cormlets, circumference of cormlets, number of nodes per cormlets and rhizome weight exhibited higher values in plants grown under 50 % shade. However, number of tiller, number of cormlets and length of internodes were found more in plants grown in large bed under field conditions (Table 71). Freitez *et al.* (2003) reported that all the evaluated variables of ginger plants were superior in partial shade, independently of the type of propagation (*in vitro* and conventional).

Zhenxian *et al.* (2000) reported that under shaded condition, ginger leaves sustain a higher photosynthetic efficiency and high dry matter accumulation, thereby increasing the unit area yield. Earlier reports revealed that, 50 to 60 shading is suitable for better growth of ginger (Shaottui and Zhenxian, 1998, Xizhen *et al.*, 2001).

4.6. Path Analysis

4.6.1. Aerial stem regenerated plants

4.6.1.1. First generation plants

Correlation analysis of the aerial stem regenerated plants revealed a high, positive and significant correlation of fresh rhizome yield per plant with circumference of cormlets (0.92) followed by length of cormlets (0.87) and number of cormlets (0.76). Plant height also had a positive significant correlation with number of leaves, leaf length, leaf width, length of cormlets, circumference of cormlets, internodal length and rhizome yield. Tiller number exhibited negative correlation with all the characters studied except leaf number. The characters like tiller number (-0.8) and number of leaves (-0.03) showed negative correlation with the rhizome yield (Table 72 and Fig. 12).

Table 72. Correlation matrix for morphological and yield characters of aerial stem regenerated plants.

Character	X 1	X 2	X 3	X 4	X 5	X 6	X 7	X 8	X 9	X 10	Y 1
X 1	1.00	-0.25	0.32*	83***	0.37*	0.17	0.41**	0.34*	-0.26	0.32*	0.48**
X 2		1.00	0.03	-0.07	-0.59***	-0.57***	-0.74***	-0.71***	-0.44**	-0.42**	-0.80***
X 3			1.00	0.33*	-0.23	-0.12	0.00	-0.11	-0.20	-0.05	-0.03
X 4				1.00	0.39*	0.10	0.24	0.34*	-0.28	0.45**	0.39*
X 5					1.00	0.54***	0.57***	0.73***	0.12	0.71***	0.75***
X 6						1.00	0.59***	0.78***	0.30	0.49**	0.76***
X 7							1.00	0.79***	.61***	0.52**	0.87***
X 8								1.00	0.45**	.76***	0.92***
X 9									1.00	0.15	0.40**
X10										1.00	0.64***

* Significant at 0.05 level, ** Significant at 0.01 level, *** Significant at 0.001 level

Plant height (X 1), No. of tillers (X 2), No. of leaves (X 3), Leaf length (X 4), Leaf width (X 5), No. of cormlets (X 6), Length of cormlets (X 7), Circumference of cormlets (X 8), No. of nodes per cormlets (X 9), Length of internodes (X 10), Weight of rhizomes (Y 1).

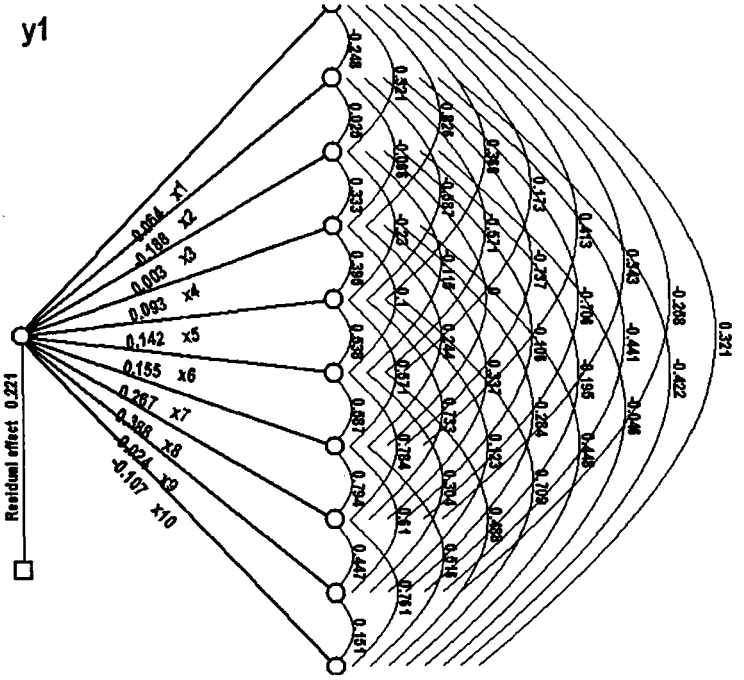


Fig. 12. Path diagram of morphological and yield characters of aerial stem regenerated plants (1st generation).

Partitioning of the correlation coefficients into direct and indirect effects revealed that circumference of cormlets followed by length of cormlets and number

of cormlets exhibited maximum positive direct effect on rhizome yield (Table 73 and Fig. 12). Maximum negative direct effect on rhizome yield was exhibited by tiller number. Circumference of cormlets through length of cormlets followed by number of cormlets, internodal length of cormlets and leaf width exhibited maximum positive indirect effect. Maximum negative indirect effect was recorded by circumference of cormlets through tiller number and length of cormlets through tiller number (Table 73). All other effects were negligible.

Table 73. Path matrix for morphological and yield characters of aerial stem regenerated plants.

Character	X 1	X 2	X 3	X 4	X 5	X 6	X 7	X 8	X 9	X 10
X 1	**0.06**	-0.02	0.02	0.05	0.02	0.01	0.03	0.02	-0.02	0.02
X 2	0.05	**-0.19**	-0.01	0.01	0.11	0.11	0.14	0.13	0.08	0.08
X 3	0.01	0.00	**0.003**	0.001	-0.00	-0.00	0.00	-0.00	-0.00	-0.00
X 4	0.08	-0.01	0.03	**0.09**	0.04	0.01	0.02	0.03	-0.03	0.04
X 5	0.05	-0.08	-0.03	0.06	**0.14**	0.08	0.08	0.10	0.02	0.10
X 6	0.03	-0.09	-0.02	0.02	0.08	**0.16**	0.09	0.12	0.05	0.08
X 7	0.11	-0.20	0.00	0.07	0.15	0.16	**0.27**	0.21	0.16	0.14
X 8	0.13	-0.27	-0.04	0.13	0.28	0.31	0.31	**0.39**	0.17	0.30
X 9	0.01	0.01	0.01	0.01	-0.003	-0.01	-0.02	-0.01	**-0.03**	-0.00
X10	-0.03	0.05	0.01	-0.05	-0.08	-0.05	-0.06	-0.08	-0.02	**-0.11**

Plant height (X 1), No. of tillers (X 2), No. of leaves (X 3), Leaf length (X 4), Leaf width (X 5), No. of cormlets (X 6), Length of cormlets (X 7), Circumference of cormlets (X 8), No. of nodes per cormlets (X 9), Length of internodes (X 10), Weight of rhizomes (Y 1).

In this case, a residual effect of 0.22 indicates that the characters studied accounted for most of the variability (Fig. 12).

4.6.1.2. Second generation plants

Correlation analysis revealed a positive significant correlation of rhizome yield with tiller number (0.50) and number of nodes per cormlets (0.27). Plant height showed positive but non significant correlation with the yield (0.04) and leaf number (0.16). However, plant height showed a positive significant correlation with leaf length (0.57) and circumference of cormlets (0.50). Tiller number exhibited positive but non significant correlation with plant height. High negative significant correlation

with the yield was observed in case of length of cormlets (-0.71) and leaf length (-0.52) (Table 74 and Fig. 13).

Table 74. Correlation matrix for morphological and yield characters of aerial stem regenerated plants – second generation.

Character	X 1	X 2	X 3	X 4	X 5	X 6	X 7	X 8	X 9	X 10	Y 1
X 1	1.00	0.11	0.16	0 .57***	0.06	-0.37*	0.17	0.50**	-0.10	0.18	0.04
X 2		1.00	-0.16	-0.23	0.10	-0.07	-0.50***	0.19	0.15	0.11	0.50**
X 3			1.00	0.33*	0.11	-0.13	0.25	0.24	0.22	0.24	-0.15
X 4				1.00	0 .34*	-0.45**	0.44**	0.29	-0.10	0.28	-0.52**
X 5					1.00	-0.10	-0.16	0.14	0.02	0.14	0.04
X 6						1.00	-0.42**	-0.06		0.28	0.24
									-0.20		
X 7							1.00	-0.23		0.05	-0.71**
									-0.12		
X 8								1.00	0.05	-0.04	0.24
X 9									1.00	0.20	0.27
X10										1.00	-0.20

* Significant at 0.05 level, ** Significant at 0.01 level, *** Significant at 0.001 level

Plant height (X 1), No. of tillers (X 2), No. of leaves (X 3), Leaf length (X 4), Leaf width (X 5), No. of cormlets (X 6), Length of cormlets (X 7), Circumference of cormlets (X 8), No. of nodes per cormlets (X 9), Length of internodes (X 10), Weight of rhizomes (Y 1).

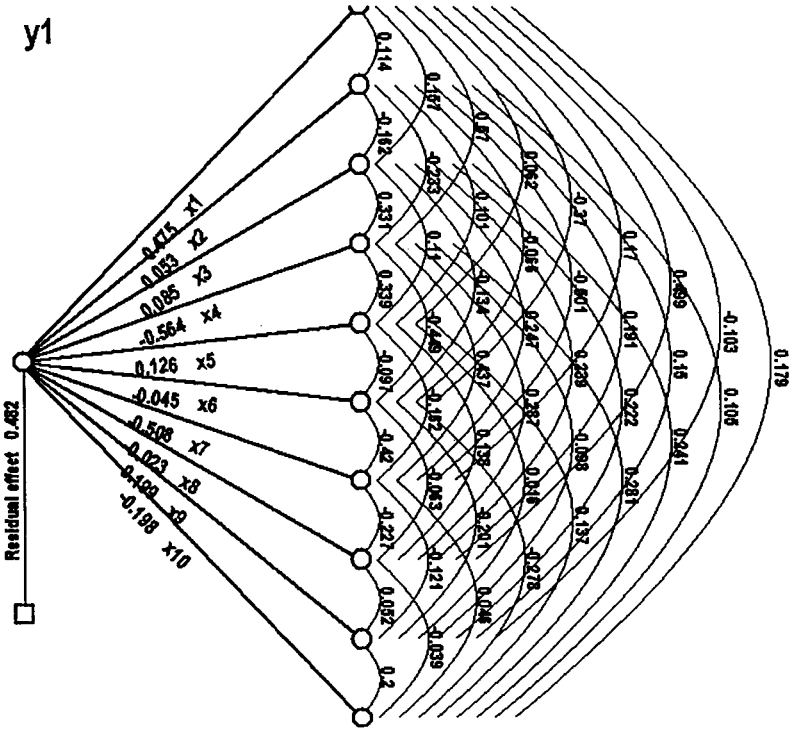


Fig.13. Path diagram of morphological and yield characters of aerial stem regenerated plants (2nd generation).

Partitioning of the correlation coefficients into direct and indirect effects revealed that characters such as plant height followed by number of nodes per cormlet and leaf width showed high positive direct effect with yield (Table 75 and Fig. 13). Leaf length and length of cormlets exhibited high negative direct effect with the rhizome yield. All other direct effects were negligible. Maximum positive indirect effect was observed with plant height through leaf length followed by length of cormlets through tiller number and leaf length through number of cormlets. Leaf length through plant height followed by leaf length through length of cormlets and length of cormlets through leaf length exhibited maximum negative indirect effect on fresh rhizome yield (Table 75).

Table 75. Path matrix for morphological and yield characters of aerial stem regenerated plants– second generation.

Character	X 1	X 2	X 3	X 4	X 5	X 6	X 7	X 8	X 9	X 10
X 1	**0.48**	0.05	0.07	0.27	0.03	-0.18	0.08	0.24	-0.05	0.09
X 2	0.01	**0.05**	-0.01	-0.01	0.01	-0.00	-0.03	0.01	0.01	0.01
X 3	0.01	-0.01	**0.01**	0.03	0.01	-0.01	0.02	0.02	0.02	0.02
X 4	-0.32	0.13	-0.19	**-0.56**	-0.19	0.25	-0.25	-0.16	0.06	-0.16
X 5	0.01	0.01	0.01	0.04	**0.13**	-0.01	-0.02	0.02	0.00	0.02
X 6	0.02	0.00	0.01	0.02	0.00	**-0.05**	0.02	0.00	0.01	0.01
X 7	-0.09	0.25	-0.13	-0.22	0.08	0.21	**-0.51**	0.12	0.06	-0.02
X 8	-0.01	-0.00	-0.01	-0.01	-0.00	0.00	0.01	**-0.02**	-0.00	0.00
X 9	-0.02	0.03	0.04	-0.02	0.00	-0.04	-0.02	0.01	**0.20**	0.04
X10	-0.04	-0.02	-0.05	-0.06	-0.03	0.06	-0.01	0.01	-0.04	**-0.20**

Plant height (X 1), No. of tillers (X 2), No. of leaves (X 3), Leaf length (X 4), Leaf width (X 5), No. of cormlets (X 6), Length of cormlets (X 7), Circumference of cormlets (X 8), No. of nodes per cormlets (X 9), Length of internodes (X 10), Weight of rhizomes (Y 1).

A residual effect of 0.48 indicates that few more characters are relevant for accounting the variation for yield (Fig. 13).

4.6.2. Vegetative bud regenerated plants

4.6.2.1. First generation plants

Association analysis of the vegetative bud regenerated plants revealed that characters like circumference of cormlets (0.92), length of cormlets (0.87), number of cormlets (0.76) and leaf width (0.75) exhibited maximum positive significant correlation with rhizome yield. Plant height also showed positive significant association with leaf length (0.83), rhizome yield (0.48), length of cormlets (0.41), leaf width (0.37), circumference of cormlets (0.34) number of leaves (0.32) and internodal length (0.32). However, tiller number showed negative correlation with all the characters except leaf number (Table 76 and Fig. 14).

Table 76. Correlation matrix for morphological and yield characters of vegetative bud regenerated plants.

Character	X 1	X 2	X 3	X 4	X 5	X 6	X 7	X 8	X 9	X 10	Y 1
X 1	1.00	-0.25	0.32*	0.83***	0.37*	0.17	0.41**	0.34*	-0.26	0.32*	0.48**
X 2		1.00	0.03	-0.07	-0.59***	-0.57***	-0.74***	-0.71***	-0.44**	-0.42**	-0.80***
X 3			1.00	0.33*	-0.23	-0.12	0.00	-0.11	-0.20	-0.05	-0.03
X 4				1.00	0.40*	0.10	0.24	0.34*	-0.28	0.45**	0.39*
X 5					1.00	0.54***	0.57***	0.73***	0.12	0.71***	0.75***
X 6						1.00	0.59***	0.78***	0.30	0.49**	0.76***
X 7							1.00	0.79***	0.61***	0.52***	0.87***
X 8								1.00	0.45**	0.76***	0.92***
X 9									1.00	0.15	0.40**
X10										1.00	0.64***

* Significant at 0.05 level, ** Significant at 0.01 level, *** Significant at 0.001 level

Plant height (X 1), No. of tillers (X 2), No. of leaves (X 3), Leaf length (X 4), Leaf width (X 5), No. of cormlets (X 6), Length of cormlets (X 7), Circumference of cormlets (X 8), No. of nodes per cormlets (X 9), Length of internodes (X 10), Weight of rhizomes (Y 1).

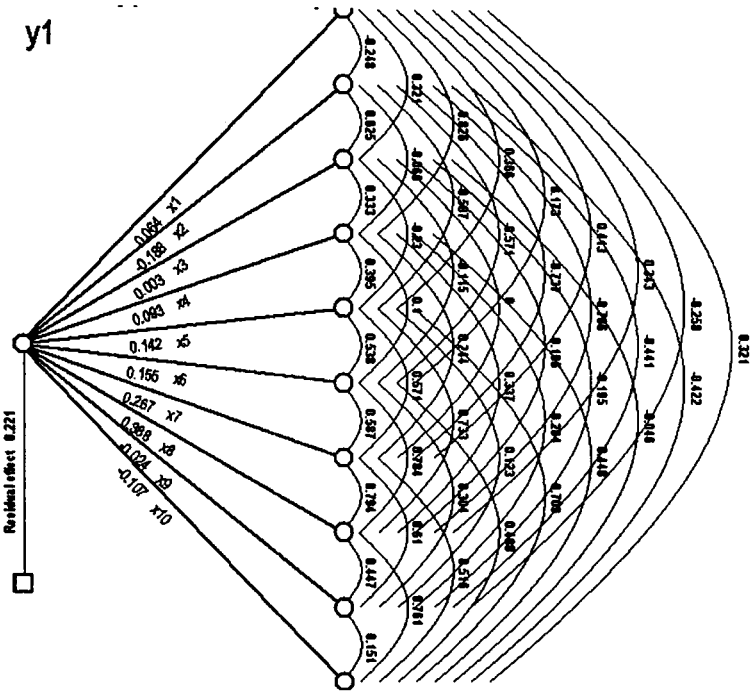


Fig.14. Path diagram of morphological and yield characters of vegetative bud regenerated plants (1st generation).

Partitioning of the correlation coefficients into direct and indirect effects showed that circumference of cormlets followed by length of cormlets showed maximum positive direct effect with rhizome yield (Table 77 and Fig. 14). Plant height too had positive direct effect on rhizome yield albeit low. But tiller number exhibited negative direct effect with rhizome yield. Circumference of cormlets through length of cormlets showed maximum indirect effect followed by circumference of cormlets through number of cormlets and internodal length. Circumference of cormlets through tiller number exhibited maximum negative indirect effect with the rhizome yield (Table 77).

Table 77. Path matrix for morphological and yield characters of vegetative bud regenerated plants.

Character	X 1	X 2	X 3	X 4	X 5	X 6	X 7	X 8	X 9	X 10
X 1	**0.06**	-0.02	0.02	0.05	0.02	0.01	0.03	0.02	-0.02	0.02
X 2	0.05	**-0.19**	-0.01	0.01	0.11	0.11	0.14	0.13	0.08	0.08
X 3	0.00	0.00	**0.00**	0.00	-0.00	-0.00	0.00	-0.00	-0.00	-0.00
X 4	0.08	-0.01	0.03	**0.09**	0.04	0.01	0.02	0.03	-0.03	0.04
X 5	0.05	-0.08	-0.03	0.06	**0.14**	0.08	0.08	0.10	0.02	0.10
X 6	0.03	-0.09	-0.02	0.02	0.08	**0.16**	0.09	0.12	0.05	0.08
X 7	0.11	-0.20	0.00	0.07	0.15	0.16	**0.27**	0.21	0.16	0.14
X 8	0.13	-0.27	-0.04	0.13	0.28	0.30	0.31	**0.39**	0.17	0.30
X 9	0.01	0.01	0.01	0.01	-0.00	-0.01	-0.02	-0.01	**-0.02**	-0.00
X10	-0.03	0.05	0.01	-0.05	-0.08	-0.05	-0.06	-0.08	-0.02	**-0.11**

Plant height (X 1), No. of tillers (X 2), No. of leaves (X 3), Leaf length (X 4), Leaf width (X 5), No. of cormlets (X 6), Length of cormlets (X 7), Circumference of cormlets (X 8), No. of nodes per cormlets (X 9), Length of internodes (X 10), Weight of rhizomes (Y 1).

A residual effect of 0.22 indicates that the characters studied accounted for most of the variability (Fig. 14).

4.6.2.2. Second generation plants

Correlation analysis revealed that leaf width (0.45) followed by number of nodes per cormlets (0.42) and number of cormlets (0.26) exhibited positive and significant correlation with rhizome yield. Plant height and tiller number also had a positive but non significant correlation with the rhizome yield. However, Plant height had a significant positive correlation with leaf length (0.70), circumference of cormlets (0.49) and leaf number (0.36). Plant height exhibited a positive but non significant correlation (0.17) with tiller number. Negative correlation of yield was observed with circumference of cormlets (-0.09) and leaf length (-0.08) (Table 78 and Fig. 15).

Table 78. Correlation matrix for morphological and yield characters of vegetative bud regenerated plants – Second generation.

Character	X 1	X 2	X 3	X 4	X 5	X 6	X 7	X 8	X 9	X 10	Y 1
X 1	1.00	0.17	0.36*	0.70***	0.20	-0.15	0.17	0.49**	-0.10	0.22	0.09
X 2		1.00	-0.15	0.01	-0.17	-0.05	0.14	0.08	0.23*	0.08	0.15
X 3			1.00	0.27	-0.03	0.06	0.24	0.05	-0.20	0.06	0.04
X 4				1.00	0.10	-0.02	0.11	0.58***	-0.27	0.38*	-0.08
X 5					1.00	0.34*	0.06	0.15	-0.07	0.32	0.45**
X 6						1.00	0.47**	0.13	-0.10	0.03	0.26
X 7							1.00	0.32	-0.15	-0.26	0.09
X 8								1.00	- 0.23	-0.10	-0.09
X 9									1.00	0.07	0.42**
X10										1.00	0.26

* Significant at 0.05 level, ** Significant at 0.01 level, *** Significant at 0.001 level

Plant height (X 1), No. of tillers (X 2), No. of leaves (X 3), Leaf length (X 4), Leaf width (X 5), No. of cormlets (X 6), Length of cormlets (X 7), Circumference of cormlets (X 8), No. of nodes per cormlets (X 9), Length of internodes (X 10), Weight of rhizomes (Y 1).

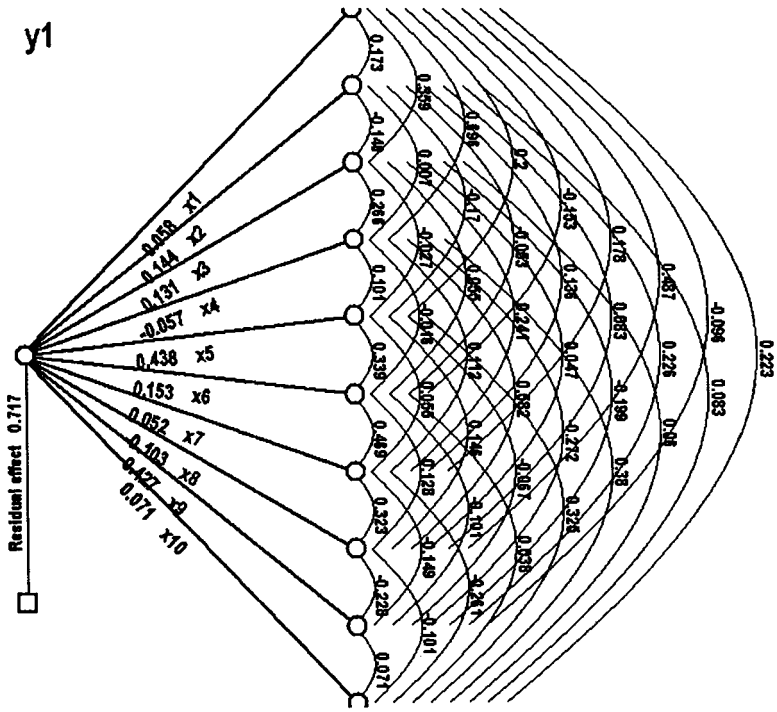


Fig.15. Path diagram of morphological and yield characters of vegetative bud regenerated plants (2nd generation).

Partitioning of the correlation coefficients into direct and indirect effects revealed that characters such as leaf width followed by number of nodes per cormlets and number of cormlets showed high positive direct effect with yield (Table 79 and Fig. 15). Tiller number and plant height also exhibited positive direct effect with rhizome yield. Maximum negative direct effect with the rhizome yield was observed with circumference of cormlets and leaf length. All other direct effects were negligible. Maximum positive indirect effect was observed with leaf width through number of cormlets followed by number of nodes per cormlets through tiller number and leaf width through plant height. Number of nodes per cormlets through leaf length followed by number of nodes per cormlets through circumference of cormlets and number of nodes per cormlets through number of leaves exhibited maximum negative indirect effect on rhizome yield (Table 79).

Table 79. Path matrix for morphological and yield characters of vegetative bud regenerated plants – second generation.

Character	X 1	X 2	X 3	X 4	X 5	X 6	X 7	X 8	X 9	X 10
X 1	**0.06**	0.01	0.02	0.04	0.01	-0.01	0.01	0.03	-0.01	0.01
X 2	0.03	**0.14**	-0.02	0.00	-0.02	-0.01	0.01	0.01	0.03	0.01
X 3	0.05	-0.02	**0.13**	0.04	-0.00	0.01	0.03	0.02	-0.03	0.01
X 4	-0.04	-0.00	-0.01	**-0.06**	-0.00	0.00	-0.02	-0.03	0.02	-0.02
X 5	0.09	-0.07	-0.01	0.04	**0.44**	0.15	0.02	0.06	-0.03	0.14
X 6	-0.02	-0.01	0.02	-0.00	0.05	**0.15**	0.07	0.01	-0.02	0.01
X 7	0.01	0.01	0.01	0.01	0.00	0.02	**0.05**	0.02	-0.02	-0.01
X 8	-0.05	-0.01	-0.00	-0.06	-0.02	-0.01	-0.03	**-0.10**	0.02	0.01
X 9	-0.04	0.20	-0.09	-0.12	-0.03	-0.04	-0.06	-0.10	**0.42**	0.03
X10	0.02	0.01	0.00	0.02	0.02	0.00	-0.02	-0.01	0.01	**0.07**

Plant height (X 1), No. of tillers (X 2), No. of leaves (X 3), Leaf length (X 4), Leaf width (X 5), No. of cormlets (X 6), Length of cormlets (X 7), Circumference of cormlets (X 8), No. of nodes per cormlets (X 9), Length of internodes (X 10), Weight of rhizomes (Y 1).

A high residual effect of 0.72 indicates that a major part of the variability is accounted by other characters (Fig. 15).

4.6.3. Callus regenerated plants

4.6.3.1. First generation plants

High positive significant correlation of rhizome yield was observed with circumference of cormlets (0.96) followed by cormlet length (0.93) and number of cormlets (0.92). Leaf width, number of nodes per cormlets, internodal length, plant height, leaf number and leaf length also exhibited positive significant association with rhizome yield. Plant height exhibited significant positive correlation with all the characters except tiller number, whereas number of tillers showed negative non significant correlation with rhizome yield and all other characters studied (Table 80 and Fig. 16).

Table 80. Correlation Matrix for morphological and yield characters of callus regenerated plants.

Character	X 1	X 2	X 3	X 4	X 5	X 6	X 7	X 8	X 9	X 10	Y 1
X 1	1.00	-0.08	0.68***	0 .65***	0.63***	0.58***	0 .72***	0 .68***	0.38*	0 .63***	0 .70***
X 2		1.00	-0.09	-0.10	-0.20	-0.22	-0.23	-0.21	-0.17	-0.08	-0.24
X 3			1.00	0.59***	0.55***	0.60***	0.67***	0.66***	0.57***	0.56***	0.67***
X 4				1.00	0.68***	0.46**	0.57***	0.59***	0.26	0.59***	0.65***
X 5					1.00	0.77***	0.77***	0.85***	0.66***	0.71***	0.87***
X 6						1.00	0.83***	0.95***	0.82***	0.70***	0.92***
X 7							1.00	0.88***	0.81***	0.61***	0.93***
X 8								1.00	0.80***	0.76***	0.96***
X 9									1.00	0.43**	0.77***
X10										1.00	0.73***

* Significant at 0.05 level, ** Significant at 0.01 level, *** Significant at 0.001 level

Plant height (X 1), No. of tillers (X 2), No. of leaves (X 3), Leaf length (X 4), Leaf width (X 5), No. of cormlets (X 6), Length of cormlets (X 7), Circumference of cormlets (X 8), No. of nodes per cormlets (X 9), Length of internodes (X 10), Weight of rhizomes (Y 1).

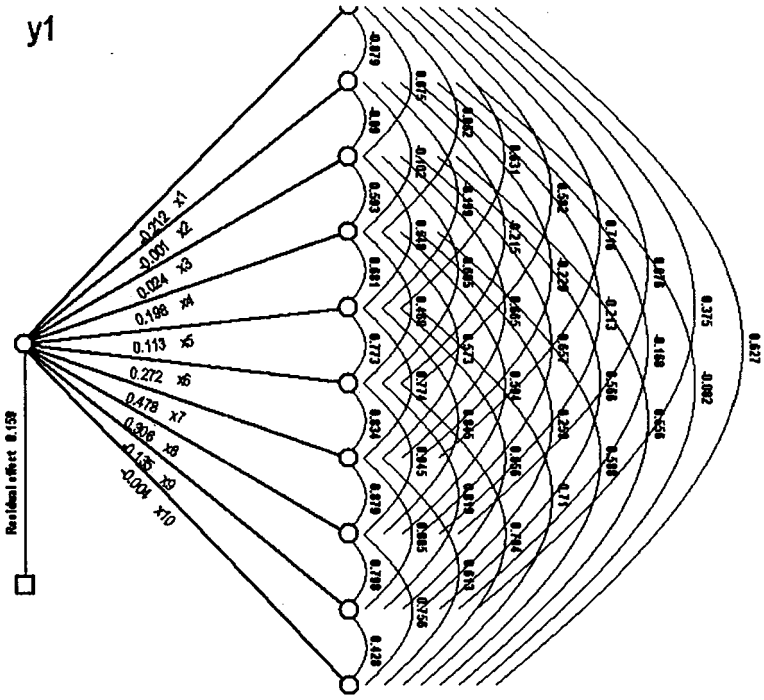


Fig.16. Path diagram of morphological and yield characters of callus regenerated plants (1st generation).

Partitioning of the correlation coefficients into direct and indirect effects revealed that length of cormlets followed by circumference of cormlets and number of cormlets exhibited maximum direct effect on rhizome yield (Table 81 and Fig. 16). Plant height, number of nodes per cormlets, internodal length and tiller number exhibited negative direct effect on rhizome yield. Length of cormlets showed good indirect effect through all the characters studied except number of tillers per plant. Number of cormlets and circumference of cormlets showed moderate indirect effect through all the characters except tiller number. Plant height through length of cormlets followed by plant height through circumference of cormlets exhibited maximum negative indirect effect with rhizome yield. All other effects were negligible (Table 81).

Table 81. Path matrix for morphological and yield characters of callus regenerated plants.

Character	X 1	X 2	X 3	X 4	X 5	X 6	X 7	X 8	X 9	X 10
X 1	**-0.21**	0.01	-0.14	-0.18	-0.13	-0.12	-0.15	-0.14	-0.08	-0.13
X 2	0.00	**-0.00**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
X 3	0.02	-0.00	**0.02**	0.01	0.01	0.02	0.02	0.02	0.01	0.01
X 4	0.17	-0.02	0.11	**0.19**	0.13	0.09	0.11	0.12	0.05	0.12
X 5	0.07	-0.02	0.06	0.07	**0.11**	0.09	0.09	0.10	0.07	0.08
X 6	0.15	-0.05	0.16	0.13	0.21	**0.27**	0.23	0.26	0.22	0.19
X 7	0.34	-0.12	0.32	0.27	0.37	0.40	**0.48**	0.42	0.38	0.29
X 8	0.21	-0.07	0.20	0.18	0.26	0.29	0.27	**0.31**	0.25	0.23
X 9	-0.05	0.02	-0.08	-0.03	-0.09	-0.11	-0.11	-0.11	**-0.14**	-0.06
X10	-0.00	0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	**-0.00**

Plant height (X 1), No. of tillers (X 2), No. of leaves (X 3), Leaf length (X 4), Leaf width (X 5), No. of cormlets (X 6), Length of cormlets (X 7), Circumference of cormlets (X 8), No. of nodes per cormlets (X 9), Length of internodes (X 10), Weight of rhizomes (Y 1).

A residual effect of 0.16 indicates that barring a 16 % of variability, most of the variability is accounted by the characters studied (Fig. 16).

4.6.3.2. Second generation plants

Association analysis revealed that circumference of cormlets (0.90) showed maximum positive significant correlation with yield followed by length of cormlets (0.89), plant height (0.85) and number of cormlets (0.84). All other characters showed moderate positive significant correlation. Plant height and tiller number showed positive significant correlation with all the characters studied (Table 82 and Fig. 17).

Table 82. Correlation matrix for morphological and yield characters of callus regenerated plants – Second generation.

Character	X 1	X 2	X 3	X 4	X 5	X 6	X 7	X 8	X 9	X 10	Y 1
X 1	1.00	0.73***	0.60***	0.75***	0.72***	0.67***	0.84***	0.80***	0.68***	0.45**	0.85***
X 2		1.00	0.47**	0.46**	0.66***	0.61***	0.70***	0.73***	0.59***	0.44**	0.69***
X 3			1.00	0.51***	0.64***	0.50**	0.66***	0.68***	0.35*	0.08	0.61***
X 4				1.00	0.46**	0.37*	0.46**	0.66***	0.36*	0.27	0.55***
X 5					1.00	0.66***	0.67***	0.79***	0.49**	0.37*	0.69***
X 6						1.00	0.78***	0.84***	0.72***	0.36*	0.84***
X 7							1.00	0.86***	0.82***	0.31	0.89***
X 8								1.00	0.72***	0.42**	0.90***
X 9									1.00	0.43**	0.76***
X10										1.00	0.50***

* Significant at 0.05 level, ** Significant at 0.01 level, *** Significant at 0.001 level

Plant height (X 1), No. of tillers (X 2), No. of leaves (X 3), Leaf length (X 4), Leaf width (X 5), No. of cormlets (X 6), Length of cormlets (X 7), Circumference of cormlets (X 8), No. of nodes per cormlets (X 9), Length of internodes (X 10), Weight of rhizomes (Y 1).

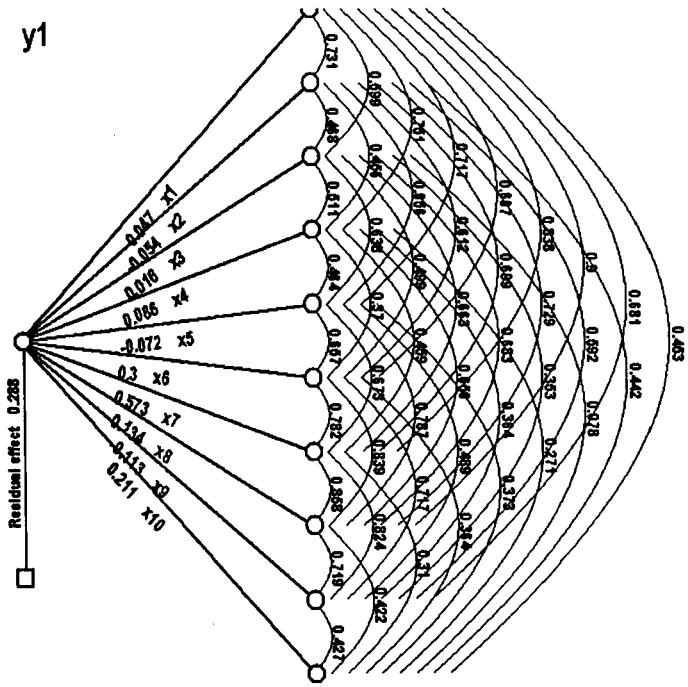


Fig.17. Path diagram of morphological and yield characters of callus regenerated plants (2nd generation).

Partitioning of the correlation coefficients into direct and indirect effects showed that characters such as length of cormlets followed by number of cormlets, internodal length of cormlets and circumference of cormlets showed maximum positive direct effect with rhizome yield (Table 83 and Fig. 17). Plant height also showed positive direct effect with yield but the number of tillers exhibited negative direct effect with yield. Length of cormlets through circumference of cormlets showed maximum positive indirect effect with yield followed by length of cormlets through plant height. Number of nodes per cormlets through length of cormlets showed maximum negative indirect effect followed by number of nodes per cormlets through circumference of cormlets (Table 83).

Table 83. Path matrix for morphological and yield characters of callus regenerated plants – Second generation.

Character	X 1	X 2	X 3	X 4	X 5	X 6	X 7	X 8	X 9	X 10
X 1	**0.05**	0.03	0.03	0.03	0.03	0.03	0.04	0.04	0.03	0.02
X 2	-0.04	**-0.05**	-0.03	-0.02	-0.04	-0.03	-0.04	-0.04	-0.03	-0.02
X 3	0.01	0.01	**0.02**	0.01	0.01	0.01	0.01	0.01	0.01	0.00
X 4	0.07	0.04	0.04	**0.09**	0.04	0.03	0.04	0.06	0.03	0.02
X 5	-0.05	-0.05	-0.05	-0.03	**-0.07**	-0.05	-0.05	-0.06	-0.04	-0.03
X 6	0.20	0.18	0.15	0.11	0.19	**0.30**	0.23	0.25	0.21	0.11
X 7	0.48	0.40	0.38	0.26	0.39	0.45	**0.57**	0.49	0.47	0.18
X 8	0.12	0.10	0.10	0.09	0.11	0.11	0.12	**0.13**	0.10	0.06
X 9	-0.08	-0.07	-0.04	-0.04	-0.06	-0.08	-0.10	-0.08	**-0.11**	-0.05
X10	0.10	0.09	0.02	0.06	0.08	0.08	0.07	0.09	0.09	**0.21**

Plant height (X 1), No. of tillers (X 2), No. of leaves (X 3), Leaf length (X 4), Leaf width (X 5), No. of cormlets (X 6), Length of cormlets (X 7), Circumference of cormlets (X 8), No. of nodes per cormlets (X 9), Length of internodes (X 10), Weight of rhizomes (Y 1).

A residual effect of 0.29 indicates that a good part of the variability (72%) is accounted by the different characters studied (Fig. 17).

In the first generation crop, circumference of cormlets, length of cormlets and number of cormlets showed high positive correlation and maximum positive direct effect with rhizome yield in all types of tissue cultured plants (aerial

stem derived, vegetative bud regenerated plants and callus regenerated plants). The tiller number exhibited negative correlation and negative direct effect with the rhizome yield. However, in the second generation of the aerial stem regenerated plants, tiller number, number of nodes per cormlets, circumference of cormlets, number of cormlets and plant height exhibited high positive correlation and maximum direct effect with rhizome yield as observed in the conventionally propagated ginger plants. These results indicate that the aerial stem regenerated plants tends to exhibit similarity with the control plants from the second generation itself.

Rathnambal *et al.* (1980) reported that when number of tiller increased the yield also increased correspondingly in the conventionally propagated plants. However, in the present study, the weight of rhizomes was not proportionally increased with increase in tiller number in the first generation crops of tissue culture plants. Tissue cultured plants showed more number of tillers as compared to control plants (Smith and Hamil, 1996; Freitez *et al.*, 2003).

Earlier reports revealed that plant height, tiller number, leaf number, width of leaves, rhizome width, rhizome length and thickness of secondary rhizome had maximum positive correlation and maximum positive direct effect with rhizome yield in conventionally propagated plants (Mohanty and Sarma, 1979; Pandey and Dhobal, 1993; Sasikumar *et al.*, 1992; Das *et al.*, 1999; Singh, 2001; Tiwari, 2003; Abraham and Latha, 2003). Panja *et al.* (2002) reported similar result in turmeric (*Curcuma longa* L.).

In the second generation of the vegetative bud regenerated plants, leaf width, number of nodes per cormlets and number of cormlets exhibited high positive correlation and maximum direct effect with rhizome yield. Tiller number exhibited negative correlation with the rhizome yield in the first generation but tiller number and plant height showed moderate positive correlation and direct effect with rhizome yield in the second generation. The present study confirms that the performance of the

vegetative bud regenerated plants were more or less comparable with the control plants.

However, in the second generation of the callus regenerated plants, circumference of cormlets, length of cormlets, plant height and number of cormlets showed maximum positive correlation and direct effect with rhizome yield. Tiller number exhibit negative correlation and negative direct effect with yield in the first generation. However, in the second generation tiller number exhibited positive correlation but negative direct effect with rhizome yield. This result indicates that the callus regenerated plants takes atleast 3 crop seasons to produce rhizome of normal size.

4.7. Quality Evaluation

4.7.1. Dry recovery

The dry recovery percentage varied significantly with varieties and explants. The effect of variety X explant interaction was also found significant (Table 84).

Table 84. Analysis of variance for dry recovery and essential oil content in ginger.

Source	Degrees of freedom	Dry recovery			Essential oil		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Replication	4	3.91	0.98	1.26^{ns}	1.84	0.46	2.66^{ns}
Variety	1	3.98	3.98	5.14*	27.08	27.08	157.16**
Explant	3	95.67	31.89	41.19**	2.89	0.97	5.60**
Variety X Explant	3	8.09	2.70	3.49*	1.22	0.41	2.36^{ns}
Error	28	21.68	0.77		4.82	0.17	
Total	39	133.33			37.85		

Mean values of dry recovery percentage of rhizomes derived from the different explants and the control plants are given in the Table 85. Among the two varieties tried, the var. Jamaica showed high dry recovery (26.1%) as compared to the var. Varada (25.2%). The mean values for explants showed that the rhizomes of

callus regenerated plants showed high dry recovery followed by the rhizome of vegetative bud regenerated plants. In both the varieties, the rhizomes of callus regenerated plants showed high dry recovery (var. Jamaica: 27.8%; var. Varada: 28.1%) followed by the rhizome of vegetative bud regenerated plants (var. Jamaica: 27.7%; var. Varada: 25.7%) and the rhizomes of aerial stem regenerated plants (var. Jamaica: 27%; var. Varada: 25.6%) (Table 85).

Samsudeen (1996) reported high dry recovery of ginger rhizomes derived from callus regenerated plants.

Table 85. Mean values for quality parameters of ginger rhizomes derived from the different explants and control.

Treatments	Dry recovery (%)			Essential oil (%)			Oleoresin (%)		
	Jamaica	Varada	Mean	Jamaica	Varada	Mean	Jamaica	Varada	Mean
RAS	27.0 (31.3)*	25.6 (30.3)	26.3	3.0 (9.9)	2.1 (8.4)	2.6	10.8 (19.1)	11.0 (19.4)	10.9
RVB	27.7 (31.7)	25.7 (30.5)	26.7	2.9 (9.8)	1.8 (7.8)	2.4	9.8 (18.2)	10.0 (18.5)	9.9
RC	27.8 (31.8)	28.1 (32.0)	28.0	2.6 (9.3)	1.7 (7.6)	2.2	8.0 (16.4)	9.1 (17.5)	8.6
RCP (control)	22.0 (27.5)	21.4 (27.9)	21.7	2.5 (9.2)	2.0 (8.1)	2.3	10.7 (19.1)	10.3 (18.7)	10.5
Mean	26.1	25.2		2.8	1.9		9.8	10.1	
CD (P=0.05)									
Variety	0.40			0.19			NS		
Explant	0.57			0.27			0.59		
Variety X Explant	0.81			NS			NS		
CV (%)	2.90			4.73			4.98		

Rhizomes from aerial stem derived plants (RAS); Rhizomes from vegetative bud derived plants (RVB); Rhizomes from callus derived plants (RC); Rhizomes from conventionally propagated plants (RCP).

*Percentage values were transformed using Arcsin √Percentage transformation table and given in the brackets.

4.7.2. Essential oil

Essential oil content varied significantly with varieties and explants but the effect of variety X explant interaction was found non significant (Table 84).

Mean values of essential oil content of the rhizomes derived from different explants and the control plants are given in the Table 85. The results revealed that the var. Jamaica showed more oil content (2.8%) as compared to the var. Varada (1.9%). Mean values for explants indicated that the rhizomes of aerial stem regenerated plants yielded more oil followed by the vegetative bud regenerated plants and the control plants. In the var. Jamaica, maximum oil content was observed in the rhizomes of aerial stem regenerated plants (3.0%) followed by the vegetative bud regenerated plants (2.9%) and the control plants registered minimum oil content (2.5%). However, in the var. Varada, maximum oil content was observed in the rhizomes of aerial stem regenerated plants (2.1%) followed by the control plants (2.0%). The rhizomes of callus regenerated plants showed minimum oil content (1.7%) (Table 85).

The var. Jamaica is reported to contain high oil (3.6%) as compared to Kurupampady (2.13 %) and Cochin ginger (2.38 %) (Rao *et al.*, 2000). Varada is a variety with moderate oil content (1.5 – 1.9 %) (Sasikumar *et al.*, 1996).

The relatively high oil content in the aerial stem regenerated plants of the two varieties may be due to the low starch and carbohydrate content present in these rhizomes (Table 87). Singh *et al.* (1991) monitored the [^{14}C] radioactivity in starch and essential oil, after exposure of the immature leaf to $^{14}CO_2$, revealed a progressive loss of radioactive label from starch and a parallel increase in radioactivity in essential oil. The results have been discussed in relation to degradation of transitory starch serving as the source of carbon precursor for essential oil biogenesis in the tissue.

4.7.3. Oleoresin

The effect of explants for oleoresin content was highly significant but the effects of varieties and variety X explant interaction were found non significant (Table 86).

Table 86. Analysis of variance for oleoresin and fibre contents in ginger

Source	Degrees of freedom	Oleoresin			Fibre		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Replication	4	3.73	0.93	1.11 ^{ns}	3.62	0.91	1.66 ^{ns}
Variety	1	0.87	0.87	1.04 ^{ns}	3.70	3.70	6.79*
Explant	3	30.45	10.15	12.11**	67.36	22.45	41.24**
Variety X Explant	3	2.77	0.92	1.10^{ns}	2.60	0.87	1.59 ^{ns}
Error	28	23.48	0.84		15.24	0.54	
Total	39	61.30					

Mean values of oleoresin content of the rhizomes derived from different explants and the control plants are given in the Table 85. Mean values for varieties revealed that the var. Varada produced more oleoresin (10.1%) as compared to the var. Jamaica (9.8%). Mean values for explants showed that the rhizomes of aerial stem regenerated plants showed high oleoresin content (Jamaica- 10.8 % and Varada – 11.0%) followed by the control plants (Jamaica- 10.7% and Varada – 10.3%). Low oleoresin content was observed in the callus regenerated plants (Jamaica- 8 % and Varada – 9.1%) (Table 85).

Rao *et al.* (2000) reported that the var. Jamaica contained 11.34 % oleoresin and it was found superior to the local ginger varieties viz. Kurupampady (7.44%) and Cochin ginger (7.43%). Sasikumar *et al.* (1996) reported 6.5 -7% oleoresin in the var. Varada. Samsudeen (1996) observed that oleoresin content was more in the micropropagated plants than in the conventionally propagated plants in the third generation.

4.7.4. Fibre

Fibre content was significantly influenced by varieties and explants but the effect of variety X explant interaction was non significant (Table 86).

The mean values of fibre content in the rhizomes derived from different explants and the control plants are given in the Table 87. The results indicated that the var. Varada had more fibre content (4.2%) as compared to the var. Jamaica (3.8%). In both the varieties, the rhizomes of control plants showed more fibre content (Jamaica - 5.2% ; Varada -5.9%) followed by the aerial stem regenerated plants (Jamaica - 3.5%; Varada – 3.7%) and vegetative bud regenerated plants (Jamaica – 3.4%; Varada – 3.6%). The least fibre content was observed in the rhizomes of callus regenerated plants (Jamaica – 2.9 %; Varada – 3.5 %) (Table 87).

Table 87. Mean values of biochemical parameters (fibre, starch and carbohydrate) of ginger rhizomes derived from the different explants and control.

Treatments	Fibre			Starch			Carbohydrate		
	Jamaica	Varada	Mean	Jamaica	Varada	Mean	Jamaica	Varada	Mean
RAS	3.5 (10.7)*	3.7 (11.0)	3.6	40.2 (41.7)	41.1 (43.3)	40.7	46.0 (52.5)	46.6 (52.9)	46.3
RVB	3.4 (10.7)	3.6 (10.8)	3.5	40.7 (42.6)	40.7 (42.7)	40.7	47.0 (53.6)	49.1 (57.0)	48.1
RC	2.9 (9.80)	3.5 (10.7)	3.2	40.6 (42.4)	42.2 (45.2)	41.4	47.0 (53.6)	47.7 (54.7)	47.4
RCP	5.2 (13.1)	5.9 (14.1)	5.6	42.6 (45.8)	43.6 (47.6)	43.1	47.4 (54.1)	49.9 (58.5)	48.7
Mean	3.8	4.2		41.0	41.9		46.9	48.3	
CD (P=0.05)									
Variety	0.34			NS			0.80		
Explant	0.48			1.2			NS		
Variety X Explant	NS			Ns			NS		
CV (%)	6.49			5.50			2.65		

Rhizomes from aerial stem derived plants (RAS); Rhizomes from vegetative bud derived plants (RVB); Rhizomes from callus derived plants (RC); Rhizomes from conventionally propagated plants (RCP).

*Percentage values were transformed using Arcsin √Percentage transformation table and given in the brackets.

Varada is a variety known to have low fibre content (3.29%). Earlier workers reported 4.8 – 11.72 % crude fibre in ginger rhizomes (Natarajan *et al.*, 1972 and Magalhaes *et al.*, 1997).

4.7.5. Starch

The starch content varied significantly with the types of explant used for the plant regeneration. But the effects of varieties and variety X explant interaction were found non significant (Table 88).

Table 88. Analysis of variance for starch and carbohydrate content.

Source	Degrees of freedom	Starch			Carbohydrate		
		Sum of squares	Mean squares	F value	Sum of squares	Mean squares	F value
Replication	4	4.91	1.22	0.36^{ns}	13.21	3.30	1.08^{ns}
Variety	1	7.74	7.74	2.27^{ns}	17.82	17.82	5.81*
Explant	3	36.73	12.24	3.59*	22.05	7.35	2.40^{ns}
Variety X Explant	3	5.31	1.77	0.52^{ns}	10.78	3.59	1.17^{ns}
Error	28	95.56	3.41		85.85	3.06	
Total	39	150.26			149.73		

Mean values of starch content in the rhizomes derived from different explants and the control plants are given in the Table 87. Among the two varieties tried, the var. Varada showed high starch content (41.9 %) as compared to the var. Jamaica (41.0%). The mean values of treatments (explants) showed that the rhizomes of control plants followed by the callus regenerated plants had high starch content. In the var. Jamaica, the rhizomes of control plants (42.6%) followed by the vegetative bud regenerated plants (40.7%) yielded more starch and the rhizomes of aerial stem regenerated plants (40.2 %) produced less starch than others. In the var. Varada, the rhizomes of control plants (43.6 %) followed by the callus regenerated plants (42.2 %) showed maximum starch and the vegetative bud regenerated plants (40.7 %) showed minimum starch content (Table 87).

Earlier workers reported 40.4 – 59 % starch content in ginger rhizomes (Natarajan *et al.*, 1972 and Haq *et al.*, 1986).

4.7.6. Carbohydrates

The carbohydrate content was varied significantly with different varieties but the effects of explants, variety X explant interaction were found non significant (Table 88).

Mean values of carbohydrate content in the rhizomes derived from different explants and the control plants are given in the Table 87. Mean values for varieties indicated that the var. Varada produced more carbohydrates (48.3%) as compared to the var. Jamaica (46.9%). Among the explants used for plant regeneration, the rhizomes of control plants followed by the vegetative bud regenerated plants showed maximum carbohydrate content. In the var. Jamaica, the rhizomes of control plants (47.4 %) followed by the vegetative bud regenerated and the callus regenerated plants (47.0 %) showed maximum carbohydrate content. In the var. Varada, the rhizomes of control plants (49.9 %) followed by the vegetative bud regenerated plants (49.1 %) showed maximum carbohydrate content. Minimum carbohydrate content was observed in the rhizomes of aerial stem regenerated plants (46.0 % and 46.6 %) in both the varieties (Table 87).

Kalpagam and Nirmala (2003) reported 60-70 % carbohydrates in conventionally propagated ginger rhizomes.

The results of qualitative analysis revealed that the var. Jamaica exhibited high performance for dry recovery and oil content while, the var. Varada showed good performance for oleoresin, starch and carbohydrate contents. The var. Varada showed high fibre content also.

In case of dry recovery, the rhizomes of callus regenerated plants exhibited high dry recovery in both the varieties. This may be due to the low fibre content present in the rhizomes of callus regenerated plants.

The oil and oleoresin contents were high in the rhizomes of aerial stem regenerated plants in both the varieties coupled with low dry recovery. The reason attribute to this may be the low starch and carbohydrate content present in these rhizomes. Goyal and Korla (2001) reported that the essential oil and oleoresin showed a negative correlation with dry matter. In the present study also dry recovery percentage of these rhizomes was low.

Biochemical parameters of ginger somaclones were studied by earlier workers (Sakamura *et al.*, 1986; Charlwood *et al.*, 1988; Sakamura and Suga, 1989; Bhagyalakshmi and Singh, 1988; Bhagyalakshmi *et al.*, 1994 and Nirmal Babu, 1997). These authors observed that the biochemical parameters were qualitatively similar but quantitatively differ from the control plants.

Sato *et al.* (1987) reported that gingerol content in field grown micropropagated ginger was lower than that of plants propagated conventionally. Samsudeen (1996) reported that extent of variation was more in morphological characters than in biochemical characters because most of the variations observed are of physiological in nature and also due to variation in planting material.

George, 1993b observed that the pattern of variability for quality traits do not follow a definite trend in tissue culture plants and it is influenced by a number of variables.

INVESTIGATION ON DIRECT *IN VITRO* SHOOT REGENERATION FROM AERIAL STEM EXPLANT OF GINGER (*Zingiber officinale* Rosc.) AND ITS FIELD EVALUATION

Thesis submitted to the

University of Calicut

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of

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(BOTANY)

by

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Summary and Conclusion

5. Summary and Conclusion

Ginger is an important tropical, herbaceous, perennial crop. The ginger rhizome is valued all over the world either as a spice or herbal medicine. Ginger normally propagates by its rhizome, with a low proliferation rate (10 -15 buds from one unit per year) and it is easily infected by soil born pathogens like *Pythium aphanidermatum,* and *P. vexans* (rhizome rot), *Ralstonia solanacearum* (bacterial wilt) and *Meliodogyne incognita* (nematode), resulting in heavy crop loss. Recombination breeding in ginger is handicapped by the absence of seed set. Though the crop is not amenable to conventional breeding, it is easily amenable to biotechnological approaches of crop improvement.

The present study is an attempt in this direction through developing successful and reproducible protocols for direct regeneration of plantlet from aerial stem, vegetative bud explants as well as indirect organogenesis from callus and somatic embryos including the basic aspects of ontogeny of direct regeneration and somatic embryogenesis, genetic fidelity of *in vitro* regenerated plants and field and quality evaluation vis-a-vis the control. Altogether 31 hormone combinations, two types of explants (aerial stem and vegetative bud) along with callus cultures of the two varieties viz. var. Varada and Jamaica were used in the study over a period of 3 years. The results of the study are summarized below:

5.1. *In vitro* studies

5.1.1. Direct regeneration of plantlets from in planta *aerial stem*

In case of direct regeneration of plantlets from *in planta* aerial stem, significant effects of varieties, hormones and variety X hormone interaction were observed. Maximum shoot and root regeneration was observed in the cultures containing TDZ and IBA (1:1; 1:0.1 mgl^{-1}) in both the varieties. Cultures containing

TDZ alone showed poor shoot and root formation. The var. Varada produced more number of shoots as compared to the var. Jamaica. However, the var. Jamaica showed maximum number of roots as compared to the other.

5.1.2. Direct regeneration of plantlets from in vitro *aerial stem*

Out of the three explant sources tried (basal, middle and top portions of *in vitro* aerial stem), basal portion containing axillary meristem followed by middle portion containing apical meristem produced maximum number of shoots and roots in both the varieties. The number of shoots and roots varied significantly with varieties and levels of hormones. The interaction between variety X hormones was significant only for shoot development. *In vitro* aerial stem produced multiple shoots in all combinations of BAP and NAA but maximum shoot and root regeneration was observed in BAP and NAA (2:1 mgl^{-1}) in the var. Jamaica and in BAP and NAA (2:1; 5:1 mgl^{-1}) in the var. Varada. Cultures containing BAP alone showed poor shoot and root development. The var. Jamaica produced more number of shoots and roots compared to the var. Varada.

The present study revealed that atleast one auxin and one cytokinin are essential for successful plantlet regeneration from the different explants of ginger. The use of cytokinin alone showed poor performance.

Direct regeneration protocol in ginger is relevant not only in the production of disease free planting materials but also in the genetic transformation studies, purification of germplasm and in germplasm exchange.

Being a monocotyledonous crop, *Zingiber officinale* Rosc. does not allow great explant diversity for micropropagation. Rhizome buds and shoot apex have been the responsive explants often used. However, rhizome explants often carries soil born pathogens. Additionally ginger rhizomes are not available during the crop season. Therefore, in the present study, aerial stem explants were tried for plantlet

regeneration, as a novel source. The *in planta* aerial stem explants were comparably less contaminated with the soil born pathogens and these were available through out the crop season. The use of *in vitro* aerial stem explants were also preferred since sterile explants could be available all the year-round.

5.1.3. Direct regeneration of plantlets from vegetative bud explants

Protocol for direct regeneration of plantlets from vegetative bud was also developed. Significant effect of hormones on shoot and root development was observed in this case. The effects of varieties and varieties X hormone interaction were non significant. Vegetative buds produced multiple shoots in all the combinations of BAP and NAA but maximum shoots and roots were observed in BAP and NAA (1:1mgl^{-1}) in both the varieties. The var. Varada produced more number of shoots and roots as compared with the var. Jamaica.

Plantlet regeneration from vegetative bud explants were carried out mainly for conducting a comparative study with the aerial stem derived plants. The rate of contamination was more in vegetative bud explant cultures compared to aerial stem cultures.

5.1.4. Callus induction

Callus induction and callus regeneration studies were also carried out. In the present study, both *in planta* and *in vitro* aerial stem explants were used for callus induction. Percentage of explants callused varied with type of explants, varieties and levels of hormones. In callusing response, the var. Varada was better than the var. Jamaica. Similarly, *in vitro* aerial stem explants produced more callus as compared to *in planta* aerial stem in both the varieties. However, both the explants showed good callusing with 2,4-D (2mgl^{-1}) in both the varieties.

Eventhough the calli were induced and multiplied in the same medium, the quality and type of the callus induced from different explants differed. *In vitro* aerial

stem produced hard, nodular and yellowish callus (Type I callus) while the *in planta* aerial stem produced white, soft and sticky callus, which is friable in nature (Type II).

5.1.5. Callus regeneration

Successful plantlet regeneration was observed in the callus obtained from *in planta* aerial stem explants (Type II). The number of shoots varied significantly with levels of hormones. The variety X hormone interaction was also found significant. However, the effects of varieties, hormones and variety X hormone interaction were non significant for number of roots. These calli produced shoots and roots in all the combinations of BAP and NAA. Maximum shoot regeneration was observed in BAP and NAA (1:1mgl^{-1}) in the var. Jamaica and BAP and NAA (2:0.5mgl^{-1}) in the var. Varada. Maximum root formation was observed in BAP and NAA (5:0.5mgl^{-1}) in both the varieties. However, Type I callus of both the varieties exhibit only rhizogenesis in all the hormone combinations throughout the study.

Callus culture has been an invariable step in monocot transformation. In many transformation protocols calli were used as direct target for micro projectile bombardment or for *Agrobacterium* infection. Eventhough callus induction and regeneration are time consuming and laborious, it can be used for inducing somaclonal variations.

5.1.6. Induction of somatic embryos

Somatic embryo induction from callus cultures of ginger was achieved in the present study. Type I callus showed only root formation in this case also. However, Type II callus showed good proliferation in the medium containing 2,4-D + BAP (1.0:0.5mgl^{-1}) in the var. Jamaica and 2,4-D + BAP (1.0:1.0 mgl^{-1}) in the var. Varada. Calli were subjected to stress for 40 to 60 days without subculturing and these desiccated calli produced good quality, white friable callus. These calli produced somatic embryos when cultured in the medium containing BAP (2 mgl^{-1}). The

somatic embryos of ginger follows all the typical stages of other monocotyledons i.e., globular, oval shaped and elongated, club shaped or cylindrical stages.

5.1.7. Germination of somatic embryos

Germination of somatic embryos was observed in the medium containing BAP + NAA in different concentrations. However, the number of shoots varied significantly with the levels of hormones. The variety X hormone interaction was also found significant for shoot development, whereas, the effects of varieties, hormones and variety X hormone interaction were non significant for number of roots. The optimum concentration for shoot regeneration was BAP + NAA (2.0:0.2 mgl^{-1}) in the var. Jamaica and BAP + NAA (2.0:1.0 mgl^{-1}) in the var. Varada. In case of root formation, the var. Jamaica showed good performance in BAP + NAA (1.0:0.5 mgl^{-1}) and in the var. Varada maximum root formation was observed in BAP + NAA (1.0:1.0 and 2.0:1.0mgl^{-1}).

5.1.8. Secondary somatic embryogenesis

Secondary somatic embryogenesis was observed when the embryogenic calli with somatic embryos transferred onto the medium with or without growth hormones. It was found that the presence of BAP in the medium increased the number of somatic embryos.

5.1.9. Direct somatic embryogenesis

Direct somatic embryogenesis form aerial stem and leaf explants were observed in the present study. The *in planta* aerial stem explants of the var. Jamaica produced direct somatic embryos in the medium containing TDZ + IBA (0.5:1.0 mgl^{-1}), while in the var. Varada, direct somatic embryos could be induced from the leaf base explants in the medium containing TDZ (0.5 mgl^{-1}). However, these somatic embryos failed to germinate in the regeneration medium.

The information regarding direct or indirect somatic embryogenesis and its regeneration may be useful in the production of clonal variants, clonal multiplication and genetic transformation studies in ginger.

5.1.10. Shoot proliferation

Shoot proliferation of ginger plantlets, regenerated from different explants were achieved by altering the type and concentrations of hormones in the culturing medium. Plantlets regenerated from different explants (*in vitro* aerial stem, vegetative bud, callus and somatic embryos) were subcultured onto the medium containing BAP + NAA (1.0:1.0 mgl^{-1}) and very good shoot proliferation was observed. Subculturing of *in planta* aerial stem derived plantlets to the liquid medium containing TDZ + IBA (1.0:0.5 and 1.0:1.0mgl^{-1}) prior to the subculturing onto the solid medium containing BAP + NAA (1.0: 1.0 mgl^{-1}) resulted in improved shoot multiplication.

5.2. Histological studies

5.2.1. Ontogeny of shoot and root initials from in planta *aerial stem*

Ontogeny of shoot and root initials from *in planta* aerial stem of ginger was studied. The shoot initials were originated from the primary thickening meristem and apical meristem, while the root primordial from the primary thickening meristem present in the aerial stem.

5.2.2. Ontogeny of shoot and root initials from in vitro *aerial stem*

In case of *in vitro* aerial stem explants, the shoot initials originated from the primary thickening meristem, apical, axillary and accessory buds of the aerial stem. While the root initials originated from the primary thickening meristem alone.

5.2.3. Somatic embryo induction /origin of somatic embryo

The histological sections of embryogenic callus culture with somatic embryos revealed the different developmental stages of somatic embryos. The somatic

embryos were developed from single meristamatic cells which underwent continuous divisions and formed quadrant and octant stages, then formed a spherical shaped embryoid. This globular embryoid further developed to an oval shaped embryo and at the stage of maturity it attained a cylindrical shape with a scutellum, coleoptile, shoot apex and a coleorhiza. The embryo also had a multicellular stalk, the suspensor.

Secondary somatic embryos were developed from the cells present in the coleorhizal part of mature primary somatic embryos.

Somatic embryos were directly induced from the primary thickening meristem of *in planta* aerial stem explant and from the epidermal tissues of leaf base explant.

5.3. Hardening

The hardening of *in vitro* plants was done with different hardening media and successful acclimatization was obtained. The hardening medium containing soil : sand : coir dust : cow dung + 5 g *Trichoderma* / cup gave good result in all aspects like survival of hardened plants, plant height, number of leaves and chlorophyll content. However, the height of hardened plantlets varied significantly with varieties and treatments. Effects of treatments and variety X treatment interaction were found significant for number of leaves, chlorophyll ^{a}, Chlorophyll ^{b} and total chlorophyll contents.

5.4. Genetic fidelity analysis

Genetic fidelity analysis of *in vitro* derived ginger plants using 18 random decamer primers revealed that the plants regenerated from aerial stem and vegetative bud explants were genetically similar with the control plants. No intraclonal variability could be observed among these plants. However, the callus regenerated plants exhibited some genetic variations in the RAPD profiles of a number of primers tested. Intraclonal variability was also observed in the callus regenerated plants.

5.5. Field evaluation

The analysis of variance for morphological and yield characteristics of field grown *in vitro* derived plants (3 years) revealed that the performance of tiller number, length of cormlets and circumference of cormlets were stable during different years of cultivation. Plant height, length of leaves, length of cormlets, circumferences of cormlets, number of nodes per cormlet and weight of rhizomes were significantly influenced by the genotype, while the effect of explants were significant for all the characters studied. The variety X explant interaction was found significant for all the characters except tiller number, while the year X explant interaction was found significant for plant height, tiller number, number of leaves, leaf width, number of nodes per cormlets and weight of rhizomes. Year X variety interaction was found significant for leaf length and number of nodes per cormlets. The interaction between year X variety X explant was found significant for plant height, circumference of cormlets and number of nodes per cormlets.

The analysis of variance for morphological and yield characteristics of second generation of *in vitro* derived plants revealed that plant height, number of leaves, leaf length, leaf width, length of cormlets, circumference of cormlets and weight of rhizomes were significantly influenced by varieties, explants and variety X explant interaction, whereas, number of tillers and length of internodes varied significantly with explants. The variety X explant interaction was also found significant in these cases. However, number of cormlets and number of nodes per cormlets were significantly influenced by varieties and explants.

The performance of varieties used in the study varied in the first and the second generations. The var. Jamaica showed good performance for the number of tillers, length of cormlets, circumference of cormlets, number of nodes per cormlet and weight of rhizomes in the first generation. However, during the second generation, this variety exhibited good performance for the characters such as tiller number, number of cormlets, number of nodes per cormlet and weight of rhizomes

whereas, the var. Varada showed better performance for plant height, number of leaves and leaf length in the first generation and during the second generation, this variety exhibited good performance for plant height, number of leaves, leaf length, leaf width, length of cormlets and circumference of cormlets.

The performance of explants used in the study also varied with the different varieties over different generations. In case of the first generation crop of the var. Jamaica, the aerial stem regenerated plants exhibited good performance for aerial morphological characters like plant height, tiller number, number of leaves and leaf length. However, for the rhizome characters, the control plants exhibited good performance. In the second generation crop, the aerial stem regenerated plants showed good performance for most of the characters like plant height, tiller number, number of leaves, leaf width, number of cormlets, circumference of cormlets, number of nodes per cormlet and weight of rhizomes.

In the first generation crop of the var. Varada, the control plants showed better performance for all the characters barring tiller number and leaf number. During the second generation crop also, the control plants registered good performance for plant height, tiller number, leaf length, leaf width, length of cormlets, circumference of cormlets and weight of rhizomes. For other traits like number of leaves, number of cormlets, and number of nodes per cormlet, the aerial stem regenerated plants exhibited better performance.

In the present study, number of tillers was high in all the three groups of micropropagated ginger plants compared to the control plants in both the generations in the var. Jamaica, whereas in the var. Varada, the control plants showed highest tiller number in the second generation crop.

Though the control plants exhibited more rhizome yield as compared to the micropropagated plants in the first generation, during the second generation itself, the

aerial stem regenerated plants produced large and bold rhizomes as compared to the control plants in the var. Jamaica. In the var. Varada, the control plants produced more rhizomes compared to others in both the generations.

The callus regenerated plants of both the varieties exhibited poor performance for all the characters studied as compared to other plants (aerial stem derived, vegetative bud regenerated and control plants).

In case of yield evaluation of plants grown in large beds, the characters such as plant height, number of leaves, leaf length, leaf width, length of cormlets, circumference of cormlets, number of nodes per cormlets and rhizome weight exhibited higher values for plants grown under 50 % shade. However, maximum number of tillers, number of cormlets and length of internodes were found in plants grown in large bed under field conditions.

5.6. Path analysis

The pattern of path analysis revealed a mixed pattern over the generations/explants.

In the first generation crops, circumference of cormlets, length of cormlets and number of cormlets showed high positive correlation and maximum positive direct effect with rhizome yield in all the types of tissue cultured plants (aerial stem derived, vegetative bud regenerated plants and callus regenerated plants). The tiller number exhibited negative correlation and negative direct effect with the rhizome yield. However, in the second generation of the aerial stem regenerated plants, tiller number, number of nodes per cormlets, circumference of cormlets, number of cormlets and plant height exhibited high positive correlation and maximum direct effect with rhizome yield as observed in the conventionally propagated plants.

In the second generation of the vegetative bud regenerated plants, leaf width, number of nodes per cormlets and number of cormlets exhibited high positive correlation and maximum direct effect with rhizome yield. Tiller number exhibited negative correlation with the rhizome yield in the first generation but tiller number and plant height showed moderate positive correlation and direct effect with rhizome yield in the second generation.

However, in the second generation of the callus regenerated plants, circumference of cormlets, length of cormlets, plant height and number of cormlets showed maximum positive correlation and direct effect with rhizome yield. Tiller number exhibited negative correlation and negative direct effect with yield in the first generation, though in the second generation tiller number had positive correlation but negative direct effect on rhizome yield.

5.7. Quality evaluation

The results of qualitative analysis revealed that the dry recovery varied significantly with varieties and explants. The variety X explant interaction was also found significant in this case. The essential oil and the fibre content were varied significantly with varieties and explants, while, oleoresin and starch content were significantly influenced by explants used for plant propagation. However, the total carbohydrate content varied significantly with varieties only.

The var. Jamaica showed high dry recovery and volatile oil content, while the var. Varada was superior for oleoresin, starch and total carbohydrate contents. The rhizomes of callus regenerated plants exhibited high dry recovery in both the varieties. The oil and oleoresin contents were high in the rhizomes of aerial stem regenerated plants in both the varieties coupled with low dry recovery.

The present study spanning over a period of 3 years revealed aerial stem of ginger can be a better source of explant for micropropagation of true to type, disease

free plantlets with good yield and quality when compared to plants derived from other means of micropropagation in ginger. A novel protocol for direct and indirect somatic embryo induction was also developed. The study also revealed the anatomical structure of aerial stem of ginger as well as the ontogeny of shoots and roots from the aerial stem for the first time. The origin and development of somatic embryos were also discussed.

This viable protocol need to be scaled up for adopting as a commercial propagation method in ginger.

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